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CHEMICAL BLISTERING: CELLULAR AND MACROMOLECULAR COMPONENTS

Final Report

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epidermal proliferation, and purified populations of basal and differentiated (mixed) cells which can be compared for sensitivity to the toxic effects of the mustard.

The experimental rationale of the project is to define by dose-response curves those parameters of toxicity which appear in the cultures at the lowest exposures to mustard, e.g., alkylation of DNA, inhibition of DNA repair and inhibition of respiration, etc., and then to determine the relevance of these effects to the cellular lesion which is associated with exposure to mustard and is a requisite for vesication in human skin in vivo. It has been determined that the effects on DNA occur at levels of exposure below those needed to cause an abnormality in the respiration of mitochondria, glycolysis, utilization of glucose, protein synthesis, or RNA synthesis. It is possible, however, that effects on metabolic and ultrastructural parameters are necessary to obtain cellular necrosis.

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SUMMARY

The overall objective of this investigation is to elucidate the molecular mechanisms by which bis-(β -chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The study was done using cultures of cutaneous keratinocytes in order to focus on the direct interactions between the mustard and the cellular targets.

The technical objectives of this project are to develop appropriate culture systems for use in the investigation of subcellular and macromolecular toxic manifestations, to establish the credibility of these systems for investigating the molecular mechanisms of these effects, and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized.

Three biological systems with which to study the molecular and cellular effects of exposure to BCES have been developed.

Protocols have been defined by which stratified terminally differentiating primary cultures of cutaneous keratinocytes derived from human or rat skin can be obtained. These cultures are grown on collagen gels or nylon membranes as substrata, with the latter being the best choice. The product is a piece of "epidermis" which is similar to the tissue *in situ* by the criteria of ultrastructure and biochemistry. The cultures have desmosomes, bundles of intermediate filaments (i.e., tonofilaments), keratohyalin-like granules, and as many as 20 cornified orthokeratotic layers on their upper surface. Biochemically, these cultures exhibit the changes in carbohydrate structure on the cell surface visualized by the binding of lectins *in situ*. Staining with appropriate monoclonal antibodies indicates that the cultures show the progression of keratin maturation seen *in vivo* as well. As yet, these biochemical parameters have only been determined on cultures grown on collagen. These stratifying terminally differentiating cultures are obtained by initial growth being submerged until confluence is reached, followed by growth at the air/liquid interface until complete cornification is achieved. The growth medium is 90% Eagles' minimum essential medium and 10% fetal bovine serum, to which are added 10 ug/ml of hydrocortisone, 10 ug/ml of insulin, and an antibiotic mixture consisting of 100 units/ml of penicillin, 100 ug/ml of streptomycin, and 0.05 ug/ml of fungizone. Since this "lifted" culture has a smooth upper surface and a consistency which can be handled for analysis, this system can be used for topical exposure experiments.

A second type of culture available for this study is a submerged monolayer of proliferating and early differentiated keratinocytes. This culture has to date been grown in plastic culture dishes (Corning Glass Works, Corning, NY). Stratification in this culture is prevented by maintaining a reduced level of calcium ion (0.08-0.10 mM) in the medium. Although this culture was reported in the literature to be composed of proliferating cells, lectin-binding studies have revealed that there are a considerable number of early spinous cells in the monolayer. This culture can be used to identify target molecules for BCES since the toxicant is always in direct contact with the target cells in this biological system.

This type of culture can be obtained from human as well as animal cells.

In developing the techniques for obtaining appropriate inocula for cell cultivation, procedures became available by which purified populations of basal cells can be obtained. These populations are about 98% pure in terms of morphology but, again, lectin-binding studies have indicated that the preparations have small percentages of cells which have the carbohydrate surface structure of spinous cells. Nevertheless, these preparations are sufficiently pure to be useful in studying the effect of BCES on the differentiative capabilities of keratinocytes. In the process of obtaining populations of basal cells, populations of the various differentiated types can be obtained as a mixture. Centrifugation in a self forming density gradient of Percoll is used to obtain these populations. To date, this preparation has only been obtained from animal cells. There seems no reason to doubt that the procedure can be applied to human cells as well.

Application of BCES to the lifted cultures can be done in 70% ethanol or dimethylsulfoxide, the latter being preferred. The solvent itself has some effect on the culture as observed biochemically but there seems to be no convenient way around this problem given the solubility and stability characteristics of BCES.

Preliminary work has been accomplished directed at identifying the most sensitive parameter of toxicity from exposure of the culture to BCES. Preliminary data suggest that lower exposures affected the integrity and metabolism of DNA more than the utilization of glucose, glycolysis, protein synthesis, RNA synthesis and the respiration of mitochondria. Exposure of a submerged monolayer culture to 64 μM BCES resulted in the insertion of so much single strand breakage in the DNA that this level was clearly cytotoxic. On the other hand effects on metabolism required an exposure of more than 100 μM BCES. At an exposure level of 5 μM BCES, DNA is still damaged but the cells have some capability of repairing their DNA.

Glutathione S-transferase and peroxidase, two enzymes which might influence the toxicity of BCES by inactivating the toxicant, were identified as present in the epidermis and were purified from whole skin.

The biological systems developed in this project are appropriate for use in determining the mechanisms responsible for the molecular and cellular manifestations of toxicity from BCES. Attention should now be focused on determining the most sensitive toxic responses and identifying those responses which are primarily responsible for those manifestations of toxicity which are requisite for vesication.

FOREWORD

The source of animal tissue for primary cultures described in this report was neonatal rats derived from the CFN strain by random mating and reared in the School of Public Health's animal facility. This facility is under the supervision of the University Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Accreditation Association for Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The source of human tissue was foreskin obtained at routine circumcisions done at Women's Hospital, The University of Michigan Medical Center, and provided without identification of the donor. The form utilized to obtain "informed consent" was the one in use by the hospital for routine circumcision. Signature on this form allows experimental use of tissues. The use of this tissue for the present project has been approved by a University Human Subjects Review Committee, and for the protection of human subjects, the investigators have adhered to applicable policies of Federal Law 45CFR46.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

A. Statement of the Problem under Study

The overall objective of this investigation is to elucidate the molecular mechanism by which bis-(β -chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The theoretical and technical advances which have occurred in cutaneous biology over the past several decades encourage the view that this objective can be achieved. It appears likely that the most productive application of the new knowledge would be in experiments involving cultures of epidermal keratinocytes. Studies *in vitro* should allow better controlled experiments and yield more reproducible data than is true *in vivo*.

The technical objectives of this project are to develop appropriate culture systems of epidermal keratinocytes for use in the study of subcellular and macromolecular toxic manifestations resulting from environmental exposure to irritant chemicals, particularly BCES, to establish the credibility of these systems for investigating the molecular mechanisms of these effects, and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized.

The specific aims which guide the study are as follows:

1. To develop *in vitro* systems which can be used to elucidate the molecular mechanisms responsible for damage in the mammalian cutaneous epidermis exposed to BCES
2. To identify changes in morphological and/or biochemical parameters (e.g., ultrastructure, structure and metabolism of DNA, enzymatic activity, and physiological function) which can be used *in vitro* as early indicators of the type of chemical destruction of the basal germinative population of cells that is associated with intraepidermal vesication such as occurs from exposure to BCES

B. Background in Literature

1. Anatomy of blistering

Application of BCES to human skin results in an initial erythema followed by blistering. Stoughton (1) has noted that in vesication "fluid accumulation is almost always secondary to fundamental damage to the cellular structures" and has defined a blister as "an abnormal accumulation of fluid, completely replacing the pre-existing tissue structure, capped by a part or all of the epidermis." The blister seen after exposure to BCES fits this description. The progression to ultimate blistering from this agent proceeds irrevocably unless the action of the chemical is neutralized within the first several minutes of exposure. Warthin and Weller (2) and Sinclair (3) noted that the process initially involves destruction of the basal and lower spinous layers of the epidermis. Presumably, the cellular destruction that produces a cavity in the tissue precedes the actual fluid accumulation. At later stages in the destructive process, the necrosis may spread to the upper spinous and granular layers as well as into the dermis.

Blistering is rare in animals and appears to be a reaction primarily seen in humans in whom it may arise as a component of various cutaneous diseases and as a result of exposure to some biological (e.g., herpesvirus), physical (e.g., ultraviolet radiation) or chemical (e.g., mustards) stressors. The blister may develop at different levels in the skin depending upon the etiologic agent involved.

2. Biochemistry of blistering

Since different vesicants produce blisters at different levels in the tissue, it would not be unexpected if the biochemistry involved were different, depending on the location of the blister.

Exposure to a mustard in vivo will cause separation at the dermal-epidermal junction, i.e., the blister appears at that level in the tissue. From a description of the time course of dermatopathological development and the time after exposure within which the process can be reversed, it is clear that the molecular course of the pathological process is set within the first 3 min of exposure in vivo. Warthin and Weller (2) found that erythema, inflammation, and vesication were not reversed by therapy (i.e., application of chlorinated lime) initiated after 3 min of exposure to BCES.

The molecular mechanism of BCES's effect is unknown, but substances in this class are powerful alkylating agents of DNA and alkylated DNA could lead to inhibition (or at least delay) of replication, to generalized breakdown of damaged DNA leading to cell death (cf 4), and to low fidelity repair resulting in mutations with consequent disruption of normal metabolic function (4, 5). Mustards can also alkylate RNA, with consequent interference in the translation of genetic information and protein (6), resulting in metabolic disruption (4). BCES, being a bifunctional mustard, can also cross-link RNA and DNA to RNA or to protein. The most important molecular target appears to be the DNA (7). BCES alkylates and cross-links at the purine bases. Alkylation of the phosphate groups in DNA can also occur. On the basis that damaged proteins can be replaced whereas damaged DNA may be irrevocably harmed, the DNA would seem to be the most important target of toxicity by BCES.

Exposure of isolated skin to a vesicant for as little as 5 min can result in the inhibition of glycolysis and respiration (8). Glycolysis is inhibited by a lower concentration of toxicant than is necessary to obtain inhibition of respiration. In the case of exposure to mustards, this effect seems to be a consequence of a reduced level of pyridine nucleotides in the cell (9, 10). Recent evidence suggests that BCES causes a lowered level of pyridine nucleotides by virtue of the stimulation of poly (ADP) polymerase (11).

Glutathione affects the toxicity of BCES because most of the BCES that enters the body is conjugated with glutathione and excreted (12). Minor urinary excretory products may include bis-cysteinylethyl sulfone and thiodiglycol formed nonenzymatically (13).

3. Repair of alkylated DNA

In bacteria, the ability to excise BCES-alkylated products from DNA is associated with increased resistance to the mustard (14). Apparently both mono- and bifunctional adducts can be removed from DNA since comparable amounts of induced repair synthesis have been observed in HeLa cells exposed to doses of BCES and the analogous half-mustard, both compounds being equally toxic (15). However, there appears to be disagreement as to the relative rates at which repair of the two types of lesions occurs (15,16). Given the mechanisms probably involved in the two repair processes, it seems unlikely that removal of the cross-links could occur as quickly as repair of monofunctional adducts (cf 7). Intuitively, it appears more likely that the cross-link would be removed one arm at a time rather than both arms simultaneously since the latter process would insert a double strand break which could be lethal. Of course, this assumes that the mechanism of repair is not simple base replacement. Detailed information on the molecular mechanisms by which BCES-induced lesions in DNA are repaired does not seem to be available.

4. Cultivation of keratinocytes

The two main types of cells in the skin are fibroblasts (dermal) and keratinocytes (epidermal). Only the keratinocytes existing at the dermal-epidermal junction can double their DNA and undergo mitosis although all nucleated keratinocytes can repair their DNA (17, 18). Fibroblasts are easily cultivated using basal medium supplemented with serum (19). Cultivation of keratinocytes requires more stringent attention. Several systems are available for cultivating keratinocytes. The best involve the use of a substratum of collagen (20, 21) or a feeder layer of irradiated fibroblasts (22). Conditioned medium is also useful (23) but not necessary (24, 25). Good growth can be obtained by supplementing the medium with growth factors such as epidermal growth factor (22, 26) and hormones (27). Vaughan, et al. (28) reported successful cultivation, passage, and increased plating efficiency of murine keratinocytes after supplementing basal medium with hydrocortisone and insulin.

Most of the cultures of keratinocytes form monolayers, with some multilayering and production of cornified layers. However, they do not reproduce structural characteristics typical of their counterparts *in situ*. There have been previous reports of stratification of rabbit, human, and rat keratinocytes with some cellular characteristics similar to intact epidermis (17, 18, 29). Lillie, et al. (30) cultured rat lingual epithelial cells at the air/liquid interface by lifting collagen-supported cultures on organ culture grids. This resulted in stratification and terminal differentiation, with organellar components similar to the parent tissue. Preliminary data from the application of this technique to primary isolates of cutaneous keratinocytes in this laboratory suggested that this procedure could be modified to produce an "epidermis" which would be useful for the purposes of the present investigation.

C. Experimental Rationale for the Investigation

A tissue culture of cutaneous keratinocytes provides a biological system in which the direct interaction of BCES with molecular and cellular elements can be studied without the systemic influences which secondarily affect the toxic manifestations. If the culture were to have the morphological and biochemical characteristics of the epidermis *in situ*, the mode of human exposure, topical application, could be mimicked experimentally and the results would probably have more credibility in terms of application to the situation *in vivo*. Furthermore, if cultures of human keratinocytes were used, the study would be as close to the "human condition" as one could get without trauma to a human volunteer. Therefore, the main system to be developed in this investigation was the stratified terminally differentiating culture of human cutaneous keratinocytes. En route, several other systems would be used, including a proliferating monolayer of keratinocytes and cultures of keratinocytes derived from the skin of the newborn rat. Additionally, an effort would be made to isolate and purify populations of basal and differentiated cells, respectively.

Molecular parameters of toxicity were to be tested in the submerged monolayer culture to establish a range of effective exposures to BCES. The intent was to determine which indicators of toxicity appeared at the lowest exposure and then to determine whether these parameters are relevant to toxicity as observed in stratified cultures after topical application of BCES at dosages equivalent to those which produce a toxic response *in vivo*.

D. Progress Report (15 September 1982 - 14 September 1985)

The specific aims of the original contract proposal and progress toward their achievement to date are as follows:

1. To develop in vitro systems which can be used to elucidate the molecular mechanisms responsible for damage in the mammalian cutaneous epidermis exposed to ECES.

a. Development of protocols for obtaining stratified, differentiated cultures of keratinocytes

1) Culture techniques

Procedures have been developed for cultivating keratinocytes at the air/liquid interface in normal calcium so as to produce a "tissue" whose morphology and biochemistry are similar to the epidermis in situ.

a) Rat cells

Epidermal basal cells were obtained from newborn rat epidermis (28) by trypsinization, purification on gradients of Ficoll (Pharmacia, Piscataway, New Jersey) and suspension in complete growth medium [90% Eagles' minimum essential medium (MEM); 10% fetal bovine serum (both from KC Biologicals, Lenexa, Kansas); 10 ug/ml of insulin and 10 ug/ml of hydrocortisone (both from Sigma Chemical Company, St. Louis, Missouri); 100 units/ml of penicillin and 100 ug/ml of streptomycin (both from Pfizer Laboratories, New York, New York), and 0.05 ug/ml of fungizone (Gibco, Grand Island, New York)] to a concentration of 10^5 cells per ml. The cells were then seeded onto a substratum and incubated submerged in growth medium until a monolayer was formed as a result of proliferation. The monolayer of keratinocytes was then lifted to the air/liquid interface using procedures appropriate to the substratum on which the cells were grown as noted below in section c, "lifting techniques".

b) Human cells

Keratinocytes from human foreskin were isolated and cultured using essentially the same methodology as reported by Vaughan et al. (28), for the isolation of epidermal keratinocytes from newborn rat skin. The tissue was sterilized by soaking in 70% alcohol for 2 min, and the subcutaneous adipose tissue was removed by scraping with a scalpel. The tissue was then cooled to 4°C and incubated with 0.25% trypsin 1:250 (Difco, Detroit, Michigan) for 14 hr at 4°C. After rinsing with cold Eagles' balanced salt solution (EBSS), the epidermis was separated from the dermis and basal cells were brushed from the underside of the epidermis using a camel's hair brush. The cell suspension was centrifuged at 60 x g for 5 min and plated on a collagen or plastic substratum.

c) Lifting techniques

Monolayer cultures of keratinocytes grown on collagen gels were lifted by one of two methods: a) cutting triangular segments of the collagen gel containing the monolayer of keratinocytes and placing the segments on organ culture grids suspended at the interface of the liquid medium and the atmosphere, or b) growing the cells on collagen gels that had been formed on nylon mesh and lifting them by removing the entire culture from the original dish and floating it on the surface of fresh medium in a second dish.

Monolayer cultures of keratinocytes grown on synthetic membranes were lifted by one of two methods: a) the organ culture method, or b) the glass fiber pad method which consisted of saturating a pre-filter pad with growth medium and placing the membrane on top of the pad so that the surface of the culture was exposed to the atmosphere and the bottom of the culture was in contact with the medium. These procedures are diagrammed in Figure 1.

Incubation of the cultures on either substratum was then continued with the cultures being fed by diffusion of nutrients through the collagen or nylon substratum.

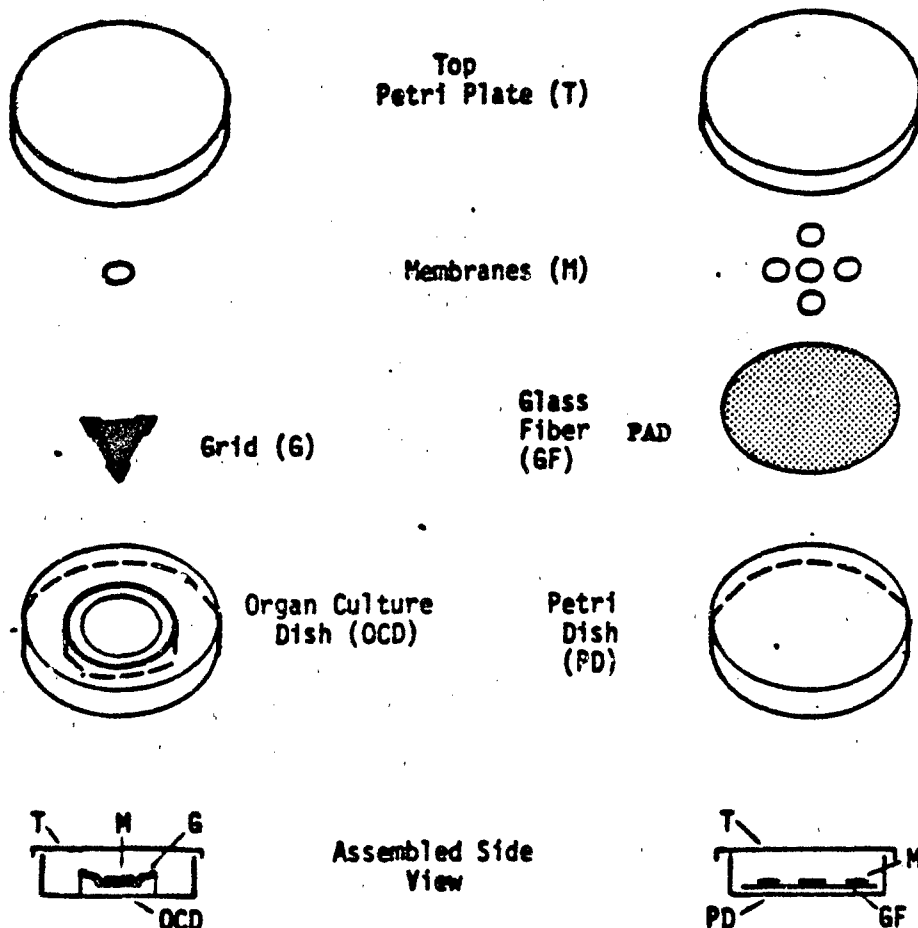
2) Studies of substrata

Different substrata were studied in an effort to identify conditions which encourage differentiation of murine keratinocytes so as to make the cells take on more characteristics of the epidermis in situ.

a) Cultivation of rat keratinocytes on collagen gels

Growth and differentiation of keratinocytes on Vitrogen 100 ~~per se~~ were unsatisfactory because the collagen gel deteriorated after 5-8 days of cultivation. The collagen substratum became thin, holes developed, and the culture contracted into clumps of cells. Attempts to alter the gel in order to overcome this problem were not successful. As a result, studies were initiated using collagens from other sources as substrata to support the cultures. As described below, combining Vitrogen 100 with other collagens provided an effective substratum for cultivation of keratinocytes from the cutaneous epidermis of the newborn rat. Other types of collagen that were investigated and found to be unsatisfactory were human placental collagen and type IV collagen from mouse sarcoma.

Rat tail and rat tendon collagens, separately, supported keratinocyte growth equally well. By 24 hr following seeding on these collagens, numerous areas of confluent growth were observed and almost three-fourths of the area of the culture vessel was covered. Confluence resulted after 4 days of incubation, as compared with 6-7 days for cultures grown on plastic. However, as a result of the low viscosity of collagen from both rat tail and rat tendon, cutting and lifting such cultures to the liquid/air interface proved to be very difficult.



METHOD I

"Organ Culture" Method

METHOD II

"Glass Fiber Pad" Method

Figure 1. Schematic diagram illustrating two methods for culturing lifted cutaneous epidermal cells at the liquid/air interface. *Method I:* The seeded cells are grown in submerged culture on the membranes for 5-7 days. A metal grid (G) is used to support the membrane on which the cells are growing. The cultures are then maintained in organ culture dishes (OCD) at the liquid/air interface for periods up to 2-3 weeks. *Method II:* The seeded cells are grown in submerged culture on the membranes for 5-7 days. The membranes are transferred to a Petrie dish (PD) containing a glass fiber filter in fresh medium, where the cultures on the membranes continue to differentiate at the liquid/air interface. The bottom diagram for each method shows a longitudinal section through both of the culture systems.

Mixing Vitrogen 100 with rat tail collagen in ratios of 1:1, 1:4, 3:1, and 4:1 (w/w) alleviated the problem of low viscosity with the latter collagen. Cells attached and spread evenly on these substrata and confluence was reached as rapidly as cells seeded on rat tail or rat tendon collagen gels. However, stratification proceeded more uniformly throughout the culture seeded on the collagen mixtures, as compared with the softer collagens. The 1:1 mixture was used to study the stratification and differentiation of keratinocytes after incubation at the air/liquid interface. Figure 2 is a cross-sectional transmission electron micrograph of a culture grown on the 1:1 collagen mixture at the air/liquid interface for 7 days. As can be seen, the cells nearest the collagen gel have a cuboidal shape with a single nucleus, one or two large nucleoli, desmosomes, tonofilaments, numerous free ribosomes, secondary lysosomes, and numerous microvilli extending from the cell surfaces. Large electron-dense inclusions resembling keratohyalin granules have also been observed. Up to 20 layers of flat, cornified cells resembling the stratum corneum have been found on the top of lifted cultures which had been incubated for 14 days at the air/liquid interface.

b) Cultivation of murine keratinocytes on synthetic membranes

1. A comparison of the ability of different membranes to support keratinocyte attachment and growth

Synthetic membranes offer a promising replacement for collagen as a substratum for the growth and differentiation of keratinocytes *in vitro*. There is good quality control for the manufacture of the membranes so that cultures should be more uniform when grown on membranes as compared with collagen gels. Various sizes can be purchased commercially or cut to specifications after receipt, thus making it much more convenient to produce raised cultures. Membranes seem to offer much more stability than collagen for the support of long-term cultivation. A major disadvantage of the membrane is its opacity. As a result, the progress of a culture cannot be followed by light microscopy, as can be done when collagen is used as the substratum. However, the advantages of using the synthetic membrane outweigh the disadvantages.

Several types of synthetic membranes have been selected for extended study. Some were selected because of their natural transparency, although most could be rendered at least partially transparent by appropriate histological procedures. Another group was examined because they were manufactured especially for tissue culture and have no added wetting agent. Some were received sterile and others were sterilized with 70% ethanol. Table 1 lists the membranes selected for study. Membranes labeled A, B, E, and F were specifically manufactured for use in tissue culture procedures.

To test the ability of these membranes to support differentiating keratinocytes, basal cells were seeded onto sterilized surfaces, submerged in medium, and incubated to produce monolayers



Figure 2. Cultured cutaneous epidermal cells on collagen (C) gel, 5 days submerged, 7 days lifted. Keratohyalin (K), suprabasal cells (SB), nucleus (Nu), stratum corneum (SC). x5,520.

TABLE 1

Synthetic Membranes Selected for Study of Attachment and Proliferation of
Rat Keratinocytes in vitro

Membrane	Pore size (μ m)	Material	Source
A. #HA-TF	0.45	Cellulose nitrate Cellulose acetate	Millipore, Bedford, Maine
B. #RA-TF	1.20	Cellulose nitrate Cellulose acetate	Millipore
C. #HV-LP	0.45	Polyvinylidene fluoride	Millipore
D. #GA-6	0.45	Cellulose triacetate	Gelman Sciences, Ann Arbor, Michigan
E. #TCM-200	0.20	Cellulose triacetate	Gelman Sciences
F. #TCM-450	0.45	Cellulose triacetate	Gelman Sciences
G. #Puropore- 200*	0.20	Nylon	Gelman Sciences
H. #Puropore- 450*	0.45	Nylon	Gelman Sciences
I. #HT-200W*	0.20	Polysulfone	Gelman Sciences
J. #Acroshield L*	0.00	Polyethylene	Gelman Sciences
K. #MEH**	1.00	Silicon-polycarbonate	General Electric, Schenectady, New York
L. #Nylon-66	0.45	Nylon	Rainin, Woburn, Maine
M. #Nuclepore	0.40	Polycarbonate	Nuclepore, Pleasanton, California

* These membranes were supplied through the courtesy of the manufacturer and are not available commercially.

** This membrane was still in the experimental stage and was obtained from General Electric by request.

as described under "culture techniques in section 1. Multiple samples of each type of membrane were seeded so that after various periods of incubation, representative cultures could be fixed in Carnoy solution, stained with hematoxylin, and mounted for light microscopy. Some cultures which exhibited good growth were converted to lifted cultures. After various periods of incubation at the liquid/gas interface, cultures were fixed and prepared for both light and electron microscopy.

The growth of keratinocytes on the various membranes was ranked qualitatively as to attachment and proliferation and compared with controls which were grown in commercially prepared plastic culture vessels. The data obtained are shown in Table 2.

The nylon membranes (G, H, and L in Table 1) supported cell growth substantially better than the others and actually promoted more than a twofold increase in cellular proliferation when compared with growth on plastic. The number of layers increased with time until about 10 days after being lifted, at which time layers near the top of the culture began to slough and the total number of layers remained more or less constant.

Membranes manufactured especially for tissue culture (A, B, E, and F in Table 1) were the next most efficient supporters of keratinocyte attachment and proliferation. The growth on these membranes also exceeded that on plastic controls. None of the naturally transparent membranes examined supported growth at any acceptable level.

Membranes selected for further study, because of resulting excellent growth on their surfaces, were the nylon and "tissue culture" grade membranes produced by Gelman Sciences, Ann Arbor, Michigan. After monolayers were formed on the surface of these membranes (7 days), the cultures were raised to the liquid/air interface to promote stratification and differentiation. Multilayering was observed for both types of membranes but more cell layers formed in cultures grown on nylon membranes than on others.

ii. Morphological observation of differentiating keratinocytes on nylon membranes

Rat keratinocytes were seeded onto nylon membranes (13 mm in diameter) placed in multiwell plastic culture vessels and incubated until a monolayer developed (6-7 days). The growth of the cells was monitored by fixing samples taken at various times and preparing them for histological examination. After the monolayer was formed, the culture growing on the membrane was lifted to the liquid/air interface by one of the two methods described above and incubated for 4-23 days, after which it was fixed for transmission electron microscopic observation.

Electron micrographs from specimens taken at selected intervals between 4 and 23 days showed that after incubating the

TABLE 2

Evaluation of Various Membranes as Substrates for Keratinocyte Attachment and Growth as Monolayers^a

Membrane	Approximate Percentage of Control Values ^b		
	24-hr Incubation	7-day Incubation	14-day Incubation
Puropore-200	150	200	250
Puropore-450	150	200	250
TCM-200	125	175	200
TCM-450	125	175	200
Nylon-66	125	175	200
HA-TF	130	175	200
RA-TF	130	150	175
HV-LP	90	80	80
GA-6	90	80	80
MEM	75	70	70
Nucleopore	60	30	30
HT-2200W	15	5	0
Acroshield L	0	0	

^a Keratinocytes, obtained as described on page 12, were seeded onto 60 mm plastic culture dishes (Corning Glass, Corning, New York) or on 47 mm membranes of the indicated materials at 2.5×10^5 cells per membrane or dish and incubated for 24 hr (indicative of attachment), or 7 or 14 days (as a measure of growth). The extent of attachment and growth was determined after fixing the cells in 10% phosphate buffered formalin, staining with hematoxylin, clearing with xylene, mounting on slides with permount and counting stained nuclei per unit area electronically using the Bioquant Image Analysis system (R. and M. Olometrics, Nashville, Tennessee), N-4-5.

^b Controls were cultures grown on the plastic dishes that contained about 900 cells/mm² after 24 hr and 1800 ± 100 cells/mm² between the seventh and fourteenth day in culture.

lifted cultures for 4 days (Fig. 3), four to five cell layers had developed, with the topmost cell layer containing degenerated nuclei. By day 7 (Fig. 4), additional layers were formed and more flat cornified cells appeared at the surface. Characteristic desmosomal attachment plaques were seen scattered throughout the cell layers. In older lifted cultures, i.e., those incubated at the liquid/air interface for 12 or 17 days (Figs. 5-7), six to seven nucleated cell layers and approximately ten layers resembling the cornified cells of the normal epidermis had formed. Several epidermal morphological markers were observed, e.g., desmosomes, keratohyalin-like granules, tonofilaments, and layers of squamous cells on the outer surface that form the stratum corneum of the epidermis in situ. The total number of cell layers appeared to have increased with time of incubation at the liquid/air interface and to have reached a maximum number at approximately 14-23 days.

Although collagen (Vitrogen 100 + rat tail collagen) can serve well as a substratum for cultivation of lifted murine keratinocytes, the nylon membrane appears to be as good if not better as a substratum. Cultures grown on the nylon membrane have more even surfaces and are better organized. Also the cells attach to the membrane more evenly than they do to collagen. The membrane is more convenient to use because of the added work and care needed to produce a collagen substratum. Unfortunately, as noted above, the membrane's opacity prevents constant monitoring of growth, which would be desirable.

c) Determination of the effect of added factors on attachment and proliferation of keratinocytes on various substrata

Several factors are employed in cell cultivation to enhance the attachment and subsequent growth of mammalian cells in vitro. Attachment factors that in vivo seem to play a role in the development of epithelial tissues have been investigated to enhance attachment and proliferation. Such factors are found in the basement membranes of these tissues and two of them, i.e., laminin and type IV collagen have been isolated in relatively pure form. Several experiments have been conducted using these factors, alone or in combination, to precoat plastic and synthetic membranes as substrata. These factors have also been compared with another biologically active factor, human fibronectin (Collaborative Research, Bedford, Maine), which is widely used in cell culture.

Laminin (Bethesda Research, Bethesda, Maryland) supported attachment of keratinocytes to both plastics and membranes much better than did human fibronectin. In fact, keratinocytes seeded on surfaces coated with the latter did not attach as well as they did on plastics while those seeded on laminin coated surfaces attached better. For example, 20% more cells attached to TCM-200 filters coated with laminin than to untreated ones although monolayers of equal confluency usually resulted in both after incubation for 7-10 days. This was determined by counting stained cells in a given growth area as described in section b) 1, above. However, a problem was discovered concerning the precoating of surfaces with laminin for cell cultivation. After about 14 days, the



Figure 3. Cutaneous epidermal cells from the rat grown on nylon membrane (M) for 4 days. Note the cuboidal shape of the cells in the basal (B) layers. Nuclei (Nu) are still retained in the uppermost suprabasal (SB) layers. x5,500.

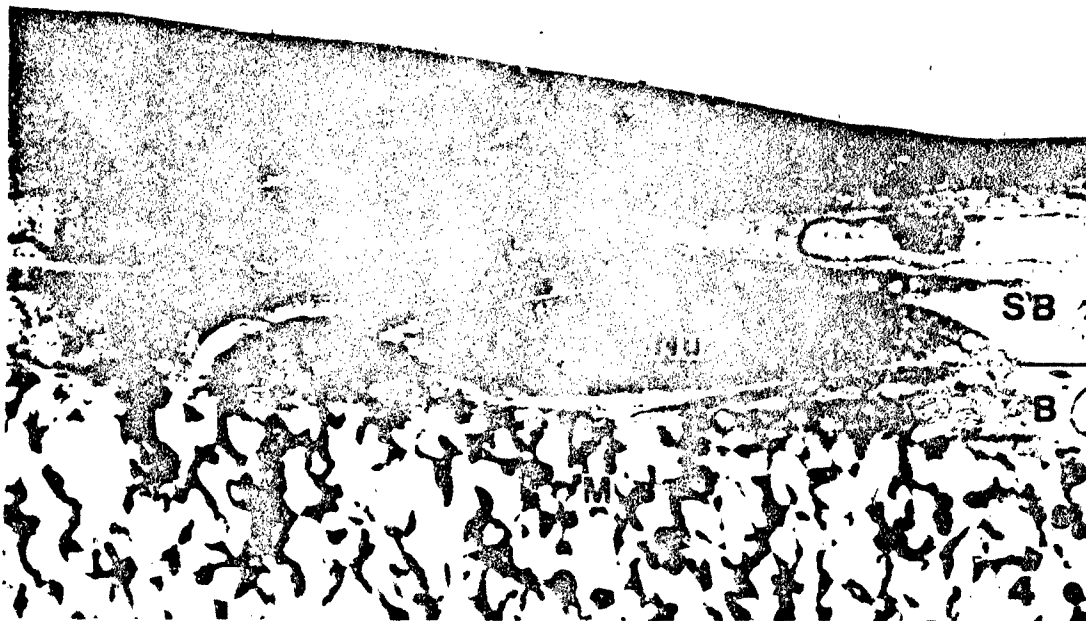


Figure 4. Cutaneous epidermal cells from the rat grown on nylon membrane (M) for 7 days. Note the flat shape of nuclei (Nu) and the lower basal (B) cells as well as the increased number of thick layers in the suprabasal (SB) strata and immature cornified (SC) cells at the top of the culture. x5,500.

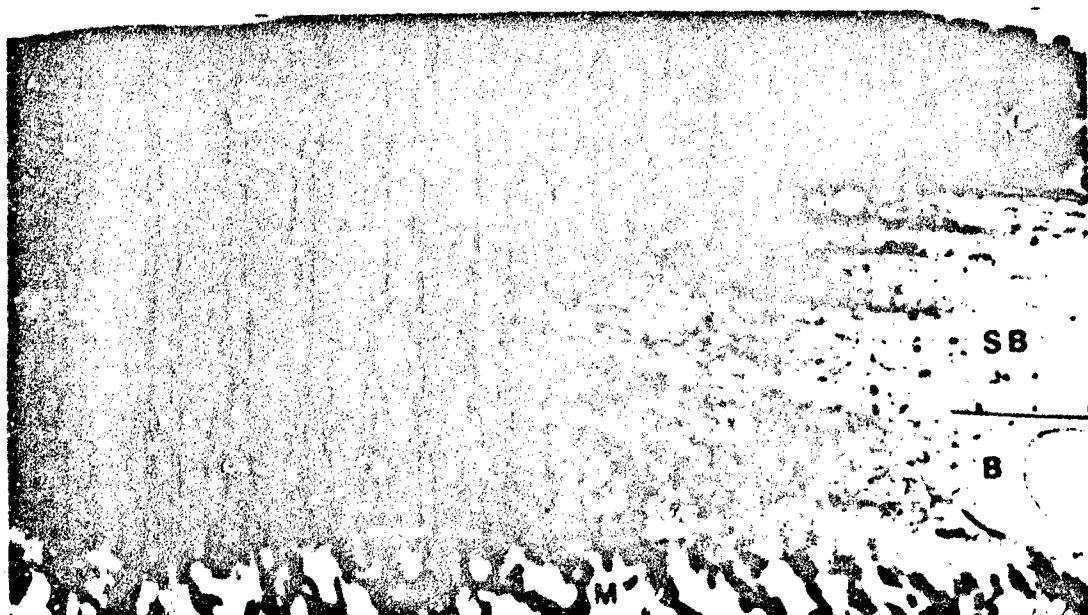


Figure 5. Cutaneous epidermal cells from the rat grown on nylon membrane (M) for 12 days. Basal cells (B), suprabasal cells (SB), stratum corneum (SC), nucleus (Nu). x5,500.

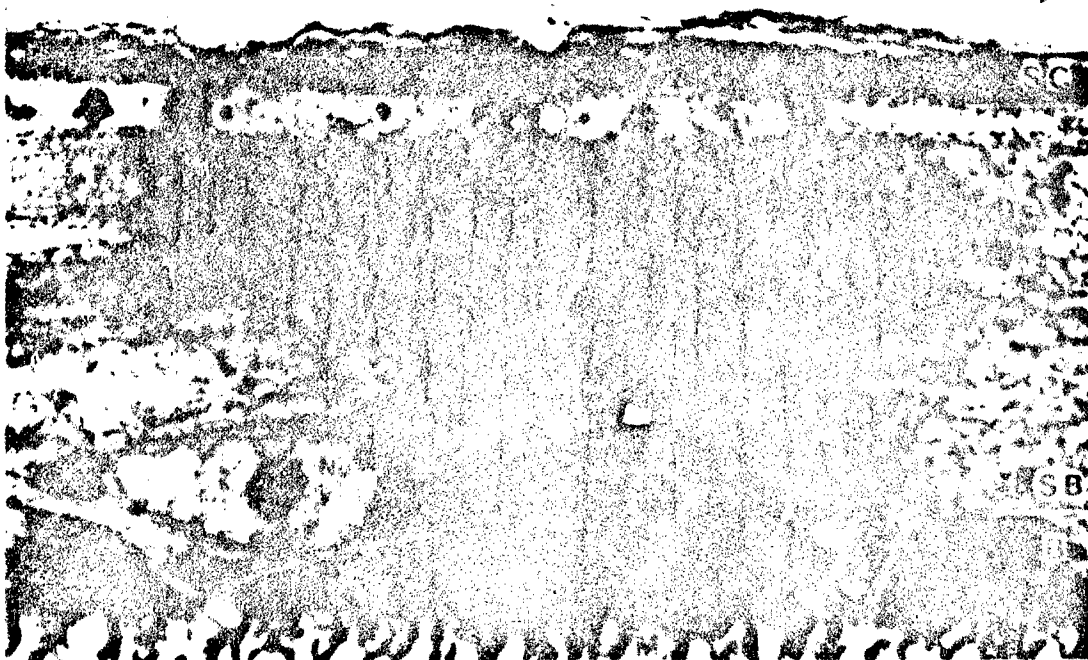


Figure 6. Cutaneous epidermal cells from the rat grown on nylon membrane (M) for 15 days. Note the accumulation of layers in the stratum corneum (SC), flat cells in the suprabasal (SB) layers, and the nucleus (Nu) in the basal layer. x5,500.



Figure 7. Cutaneous epidermal cells from the rat grown on nylon membrane (M) for 17 days. Note the presence of desmosomes (D), tonofilaments (T), and keratohyalin granules (K) in the suprabasal (SB) layers and the stratified and cornified upper layers (SC). xl,400.

culture began to detach from the surface of the substratum as a complete sheet and the culture was thus lost. We have no explanation for this phenomenon but it prevents the use of this attachment factor if long-term cultures are needed for experiments.

Type IV collagen (Bethesda Research, Bethesda, Maryland) was studied but was found to be unsatisfactory. It cannot be made into a gel but must be air dried on a surface. No increase has been observed in cell attachment using this material in this manner.

None of the attachment factors used to precoat membranes, increased attachment and proliferation of rat keratinocytes over that seen with untreated membranes. Untreated nylon membranes obtained from Gelman produced results superior to all other membranes and plastics treated or untreated.

b. Submerged low calcium cultures of murine keratinocytes

Although the stratified, cornified culture of keratinocytes is the best *in vitro* system in which to simulate the effect of topically applied BCEs *in vivo*, a monolayer of keratinocytes can be used to study the molecular interaction between mustard and cellular macromolecules. Cultured keratinocytes can be kept as a monolayer by restricting the level of Ca^{2+} in the growth medium (31). Although stratification is retarded under these conditions, differentiation does occur. As noted below, the differentiated cells seem to be primarily at the lower spinous stage of the keratinization process.

To date, monolayer cultures have been grown on plastic surfaces and not on collagen or other substrata. Populations of cells obtained by trypsinization of skin as described above were seeded into culture dishes and allowed to grow in medium consisting of calcium-free MEM supplemented with 10% Chelex-treated fetal calf serum and then adjusted to 0.11 mM Ca^{2+} with calcium chloride. Such monolayer cultures exhibited the typical cobblestone pattern of distinct cells with large intercellular spaces (Fig. 8). This culture can be maintained for more than 6 weeks and consists of both mitotically capable cells and cells which appear to be unable to divide. These may be differentiated cells since some of the cells bind the *Ulex europaeus* lectin. In tissue sections of skin, this lectin binds to the epidermal spinous cells (cf. 32). The methodology of cultivation for this preparation was worked out in another ongoing project of this laboratory. A description of the technique and the morphology of the culture has been published (33).

c. Lifted cultures of human keratinocytes

1) Cultivation of human newborn keratinocytes on collagen gels.

Attachment of human cells was satisfactory on either a collagen or plastic substratum. Small groups of round cells were seen by 2-3 days after seeding. At 5-7 days, these groups were connected by long

cellular bridges which were several cells wide. Faster growth was observed on a substratum of Vitrogen 100 than on a plastic surface. Figures 9a and 9b show the relative sizes of a particular culture after 2 and 5 days on Vitrogen 100. By 21 days, cells on Vitrogen covered about one-third of a

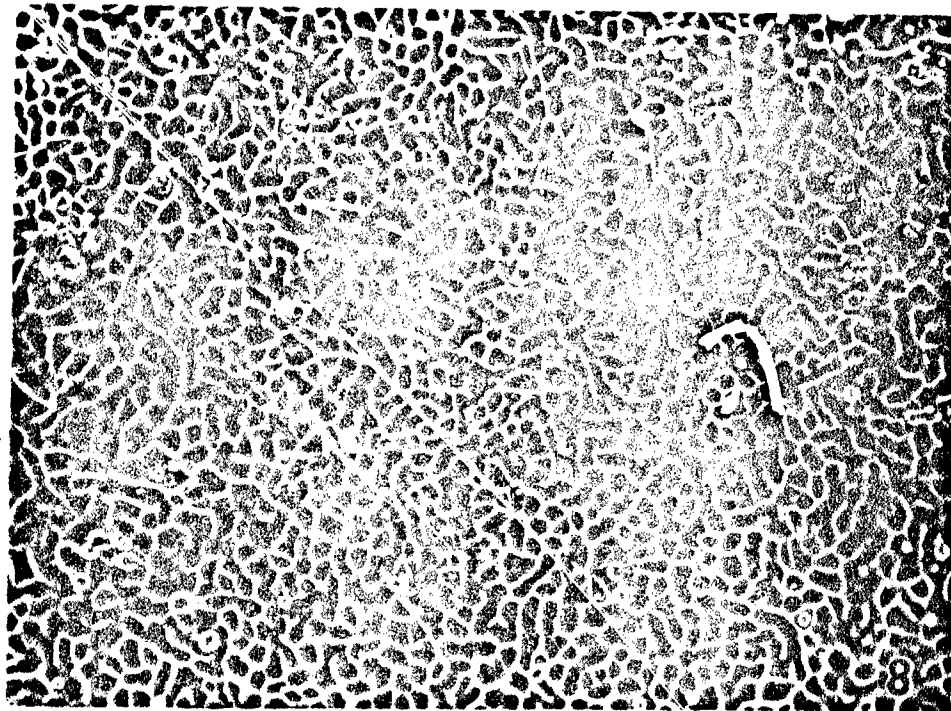


Figure 8. Phase contrast micrograph of rat keratinocytes grown for 7 days in 0.1 mM Ca^{2+} with 10% Chelex-treated fetal calf serum. x225.

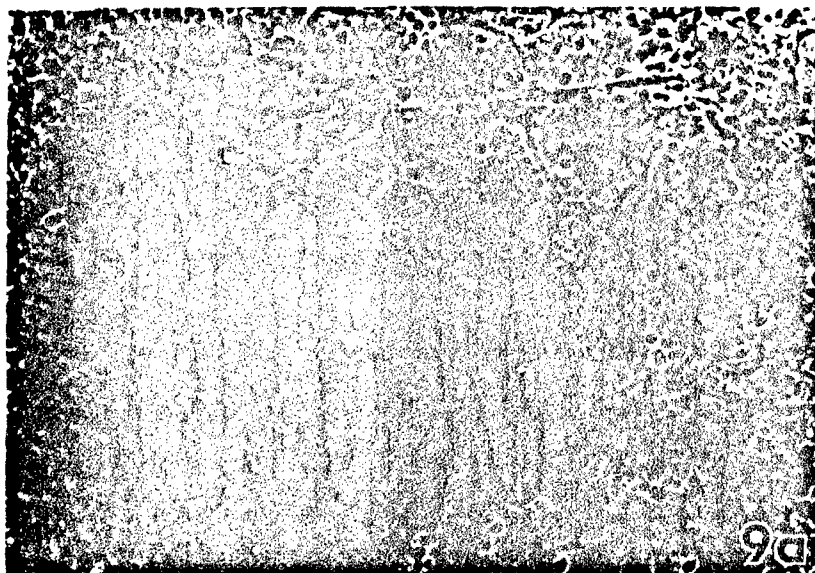


Figure 9a. Human keratinocytes grown for 48 hr on Vitrogen 100 collagen.
x205.

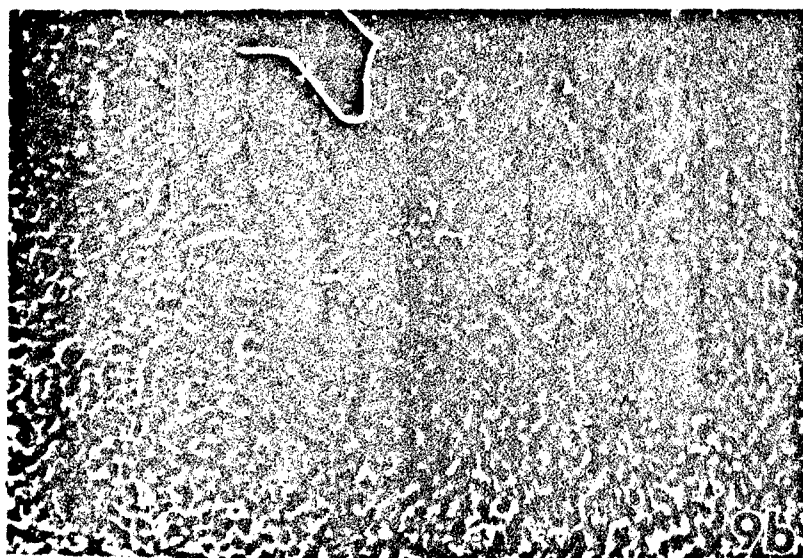


Figure 9b. Human keratinocytes grown for 5 days on Vitrogen 100 collagen.
x205.

35 mm dish. At this time, the cultures showed several layers of nucleated cells (Fig. 10a and 10b). No cornified layers were noted. After 13 days submerged, cultures were lifted and allowed to go for 8 more days. Figures 11a and 11b are light and transmission electron micrographs of the resulting cultures showing cuboidal basal cells and flat differentiated cells with typical filaments, desmosomes, and membrane-coated granules. Numerous microvilli and cytoplasmic extensions into the substratum were found. Cultures that had been grown at the liquid/air interface for 23 days showed up to 20 cornified layers (Fig. 12). In contrast to the situation with murine cells, the substratum of Vitrogen 100 did not appear to have deteriorated ever after 30 days of culture. Attempts to enhance the growth rate by using different media (e.g., 199 MEM, Ham's defined MEM, or adding 10 or 20% human serum) were not successful. Since the yield of viable cells is considerably lower from human foreskin as compared with rat epidermis, attempts were made unsuccessfully to increase the yield by effecting better dermal-epidermal separation. This was done by increasing the concentration of trypsin to 1%, trypsinizing at room temperature, lengthening the time of exposure to trypsin and using collagenase plus trypsin. It is clear that lifted cultures of human epidermal keratinocytes can be grown successfully from newborn foreskin but the efficiency of the process (i.e., the yield of cells and the time it takes to achieve the desired result) needs to be improved.

d. Development of protocols for obtaining isolated populations of purified basal cells and of mixed differentiated cells

Populations of dissociated keratinocytes from a trypsinized epidermis have been separated into two major fractions by centrifugation in a 38% self-forming gradient of Percoll (Pharmacia). A lower band in the gradient at a density exceeding 1.062 g/cc consisted of at least 98% spherical cells (Fig. 13) which exhibited a high nuclear to cytoplasmic ratio. Greater than 95% of the cells excluded trypan blue and plating efficiency was high. A less dense band covering a density range between 1.033 and 1.049 g/cc consisted of a heterogeneous population of mostly differentiated cells (Fig. 14). This population included cells with various sizes and shapes. In comparison with the spherical cells in the lower band, these cells were flat, had irregular shapes, were larger, had a lower nuclear to cytoplasmic ratio, and did not exclude trypan blue. About 5% of these cells had keratohyalin-like inclusions, suggesting that they were derived from the epidermal granular layer. Although some of these cells might attach to a substratum, they would not be expected to proliferate or remain attached for more than 24 hr (cf Vaughan and Bernstein (1971)).

e. Comparison of cytochemical parameters between epidermis *in situ* and cultures of keratinocytes.

Monolayer cultures of rat keratinocytes grown in reduced Ca^{2+} exhibit lectin binding on the cell surface which is consistent with the picture *in situ*. Brabec et al. (32) demonstrated the binding of *Bandeiraea simplicifolia* (now called *Griffonia simplicifolia*), isolectin I-B₄ (I-B₄), to the surface of epidermal basal cell and the cells of the lower two spinous layers in tissue sections of rat skin. *Ulex europaeus*

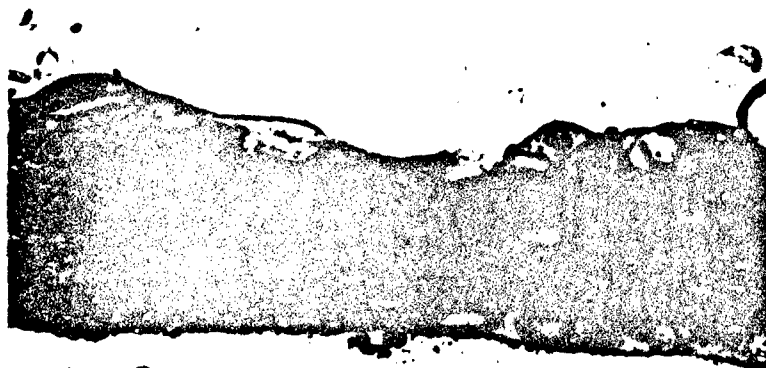


Figure 10a. Light micrograph of human keratinocytes grown submerged for 3 weeks on Vitrogen 100 collagen (C). No cornified layers are evident. x640.



Figure 10b. Electron micrograph of human keratinocytes grown submerged for 3 weeks on Vitrogen 100 collagen (C). Note tonofilaments (T) and desmosomes (D) and the absence of cornified layers. x10,000.

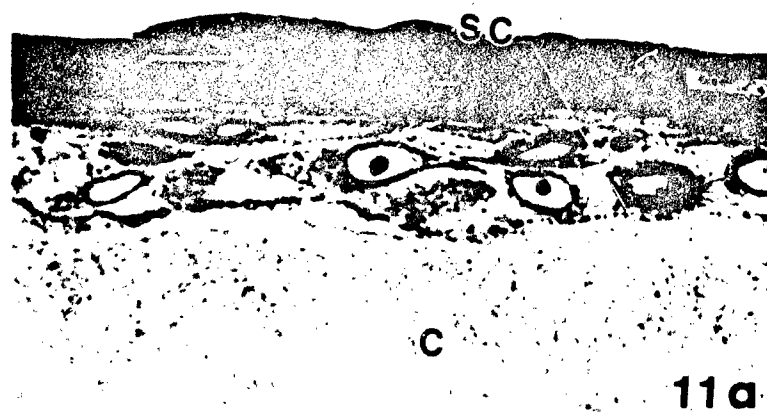


Figure 11a. Light micrograph of human keratinocytes grown submerged for 2 weeks and lifted for 8 days on Vitrogen 100 collagen (C). Note the keratohyalin-like granules (K) and the cornified layers. x640.

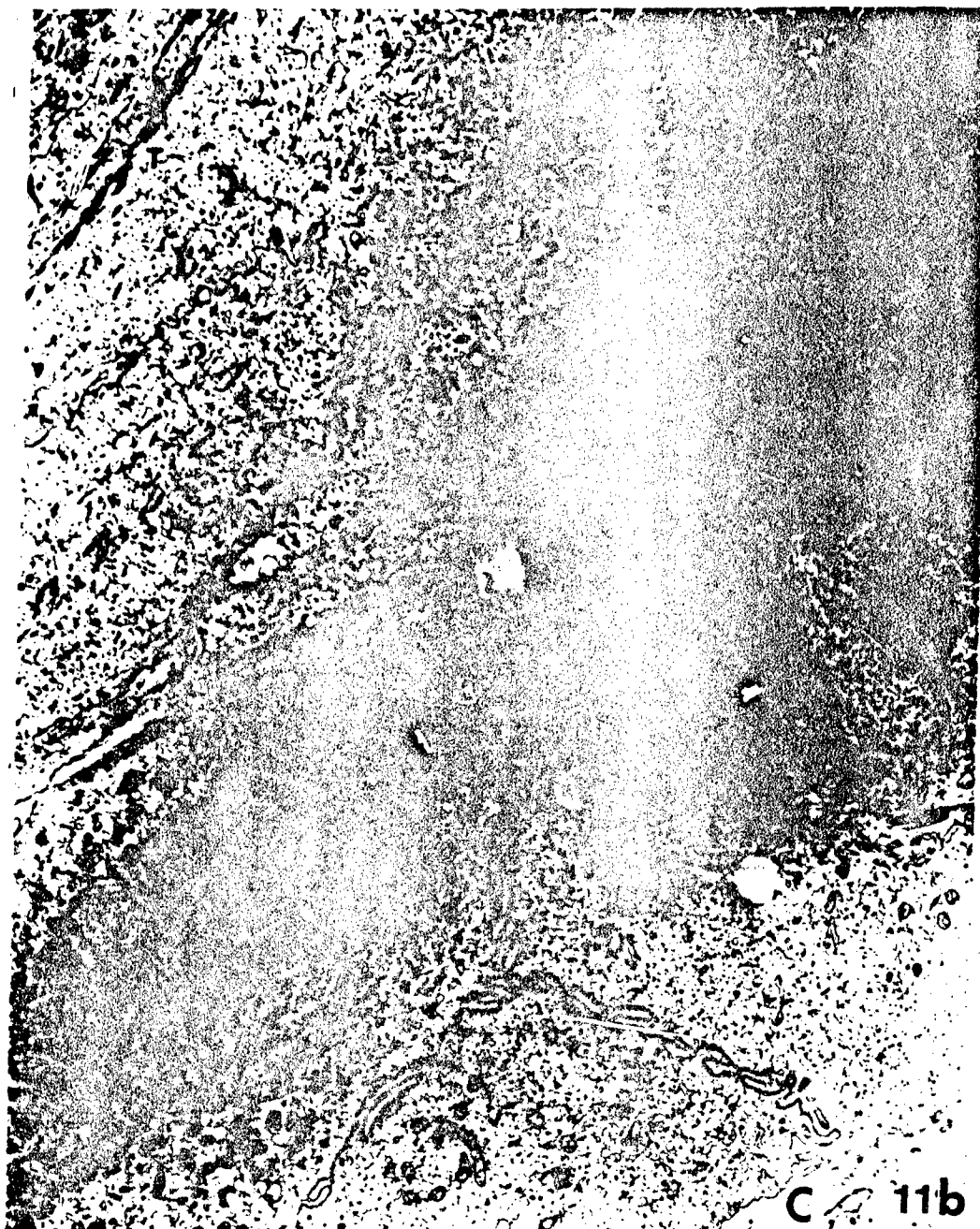


Figure 11b. Electron micrograph of human keratinocytes grown submerged for 2 weeks and lifted 8 days on Vitrogen 100 collagen (C). Note the tonofilaments (T) and desmosomes (D). x10,000.

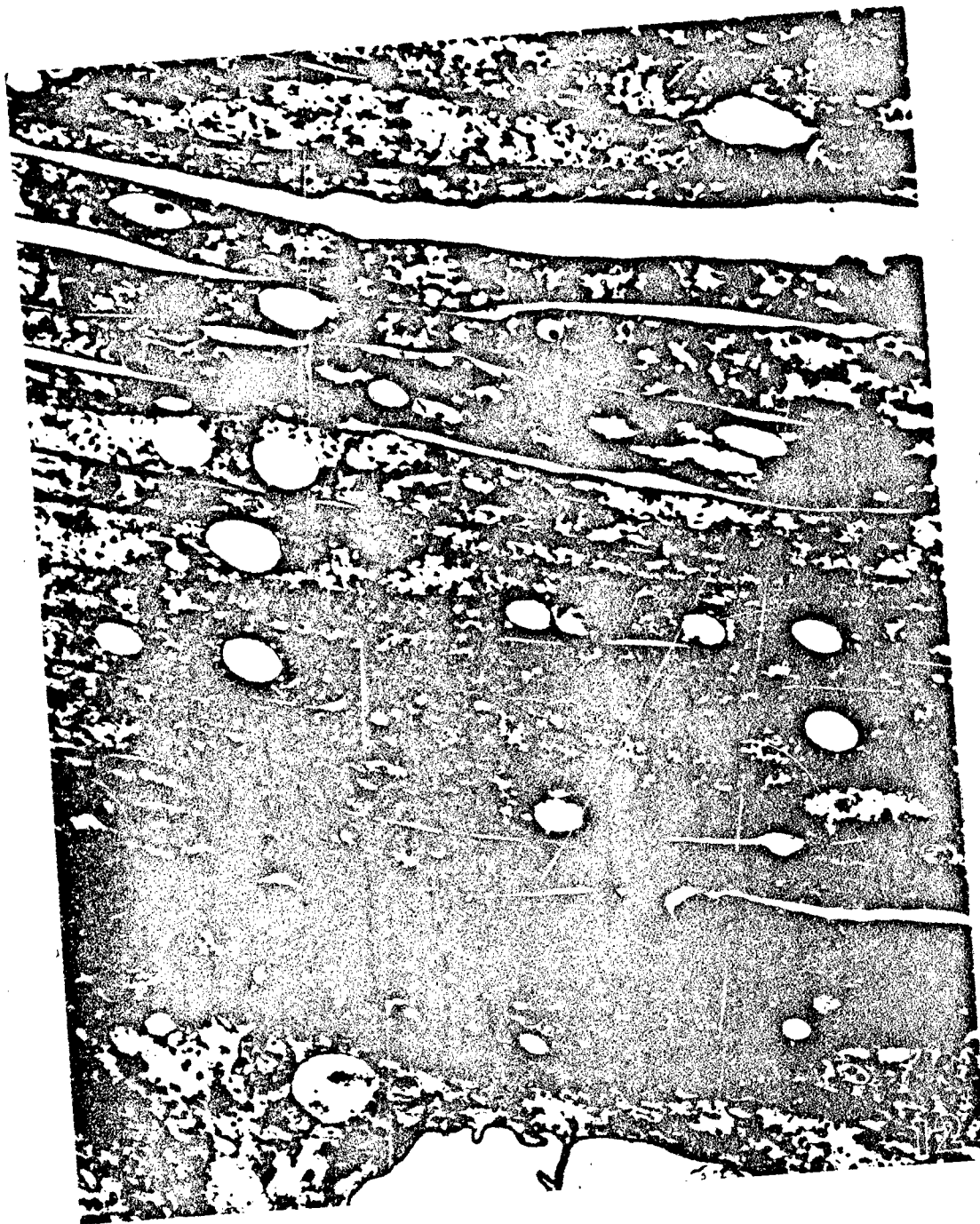


Figure 12. Electron micrograph of cornified layers of human keratinocytes grown submerged for 14 days and lifted for 23 days. x16,000.

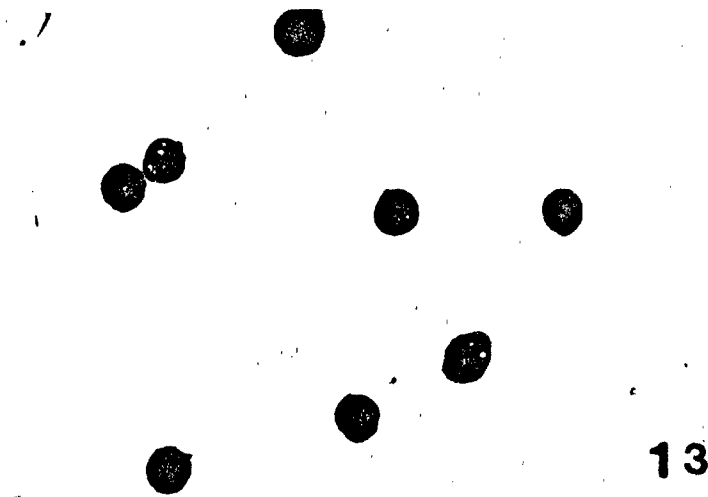


Figure 13. A smear of cells from the lowest band of a 38% Percoll gradient (density = 1.062 g/cc). Stained with Weigert's Hematoxylin. Based on their size, round structure, and dark staining, these cells were designated as basal cells. x120.

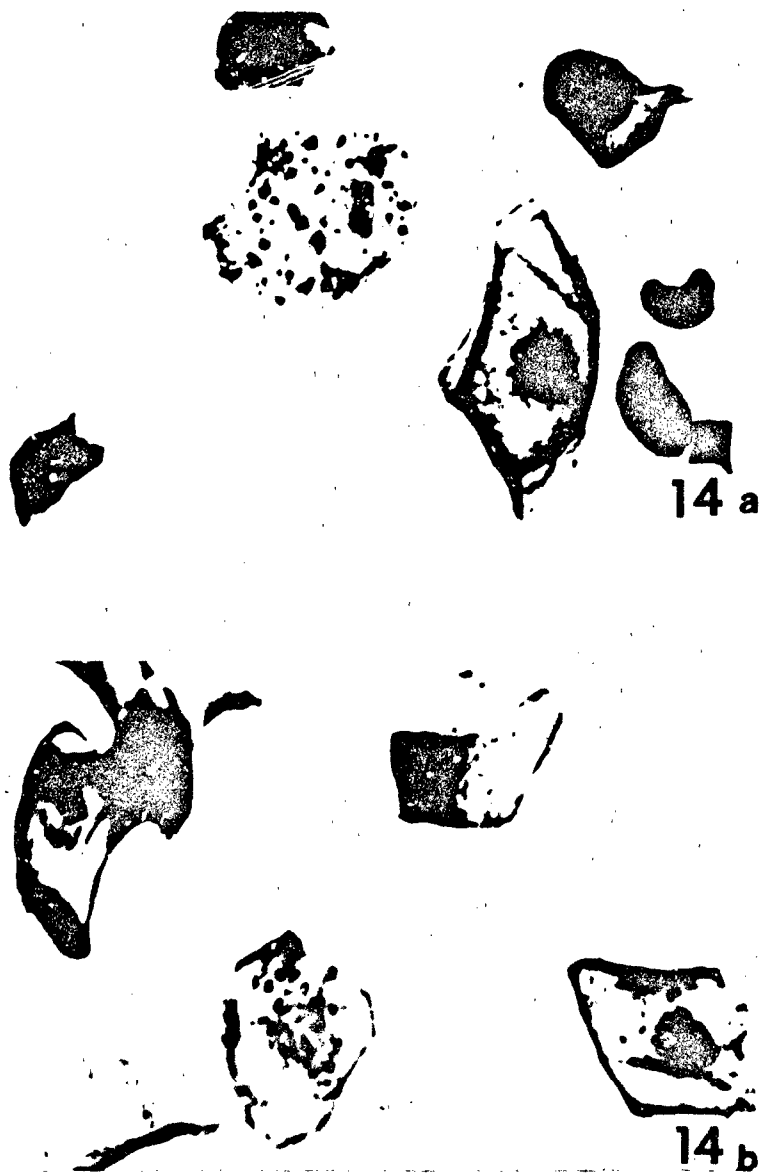


Figure 14. Smear of cells from the upper band of a 38% Percoll gradient. Density = 1.033-1.049 g/cc. Stained with Weigert's Hematoxylin. a: Note the lightly stained, irregularly shaped differentiated cells; b: Note the granular cells. $\times 120$.

agglutinin I (UEA) was found to decorate the surface of the spinous cells and not to the basal cell. Each lectin binds a surface receptor having a particular carbohydrate as the nonreducing terminal of the receptor glycoconjugate, e.g., the I-B₄ to methyl- α -D-galactoside and the UEA to α -L-fucose. In the developing monolayer culture, early during the period of cultivation, the cells were found to bind I-B₄ primarily. Later, as they differentiated, the cells also bound the UEA. In the lifted culture, GS B-I₄ decorated the basal layer and UEA, the spinous layer. Cultures grown submerged in normal Ca²⁺ (1.2 mM) show specific lectin binding in accordance with the degree of stratification and differentiation. Thus the binding of I-B₄ and UEA in culture was consistent with predictions based upon the binding of these lectins in vivo.

Lifted cultures exhibited antibody staining that was consistent with data obtained in vivo. In cultures grown submerged for 3 weeks and lifted for 3 weeks, a monoclonal antikeratin (2D6) which immunolocalizes to only basal cells of human skin bound only to cells in the basal layer of the culture. Also AE2 antikeratin (34) which localizes on granular cells in situ, did so under the cornified layer in culture.

Kinetic studies of mitosis and replication as well as repair of DNA in these cultures (untreated) are in progress.

2. To identify changes in morphological and/or biochemical parameters (e.g., ultrastructure, structure and metabolism of DNA, enzymatic activity, and physiological function) which can be used in vitro as early indicators of the type of chemical destruction of the basal, germinative population of cells that is associated with intraepidermal vesication such as occurs from exposure to BCES

a. Choice of solvent for topical application of BCES: studies of solvent effects on the cultures

A nontoxic solvent for BCES that results in uniform distribution of the agent over the surface of the lifted culture was sought. Acetone, ethanol, hexane, and methylene chloride as nonaqueous and dioxane and dimethyl sulfoxide (DMSO) as aqueous solvents were considered.

When lifted cultures were exposed to methylene chloride, ethanol, hexane or acetone by topical application for 2 min and then incubated for 0, 4, 8, and 24 hr in complete growth medium containing tritiated thymidine, the incorporation of tritium was no greater than 1% of the control value (treatment with physiological saline) during any one of the time periods. Evaporation of these organic solvents was immediate and the consequent drastic drop in temperature may have caused the irreversible cell damage which was observed as a decrease in the incorporation of tritiated thymidine. Various percentages of alcohol were also evaluated for cytotoxicity. Using the same procedure, 40% was found to be the highest alcohol concentration which the cells tolerated. However, it was observed that BCES in methylene chloride does not dissolve in 40% ethanol to the extent desirable. On the other hand, DMSO was found to be satisfactory. A comparison of the effects of the latter two solvents on the in-

corporation of labeled thymidine and leucine is shown in Table 3. The data demonstrate that exposure of differentiated cells to 70% DMSO affected metabolic activity to an extent that was similar to the effect after exposure to 40% ethanol.

DMSO was shown to be the least toxic of the effective solvents for BCES thus far examined. Experiments have been initiated to determine the effect of the solvent and various concentrations of BCES on the capacity of differentiated cultures to incorporate radioactive precursors.

TABLE 3

Incorporation of [^3H]Thymidine and [^{14}C]Leucine by Lifted Cultures After Exposure to Solvents

Treatment	Thymidine Exposure time (hr)		Leucine Exposure time (hr)	
	2.5	24	2.5	24
Untreated control	1185 \pm 279	798 \pm 125	2.69 \pm 0.6	1.87 \pm 0.19
70% DMSO	658 \pm 138	564 \pm 110	1.27 \pm 0.03	1.42 \pm 0.29
40% EtOH	836 \pm 82	542 \pm 162	2.05 \pm 0.78	1.12 \pm 0.24
Heat-killed control		83 \pm 21		0.2 \pm 0.06

Values are shown \pm standard deviation (n=3).

sors of DNA and protein. In these experiments, lifted cultures grown for 14 days were exposed to 50, 100, and 200 nmol/cm² BCES dissolved in 70% DMSO for 1 and 2 hr, pulse labeled with a labeled precursor for 0.5 hr, and then prepared for liquid scintillation counting of incorporated tracer. Figures 15 and 16 show that the incorporation of thymidine and leucine, respectively, into acid-precipitable macromolecules in relation to the time of exposure to BCES. As shown in the figures, at 1 hr after the exposure, there was an inhibition of incorporation. This inhibition increased as the dosage increased. By the second hour after exposure, the degree of inhibition had lessened. It should be noted that even the solvent control (DMSO alone) showed a similar pattern. However, in the case of the control and the lowest exposure to BCES, the level of incorporation of tracer exceeded control at the second time point. This was not true at the two highest levels of exposure. It appears that the damage occurs early after exposure to BCES and experiments are now under way to measure this effect earlier than 30 min after exposure. These experiments, although suggestive,

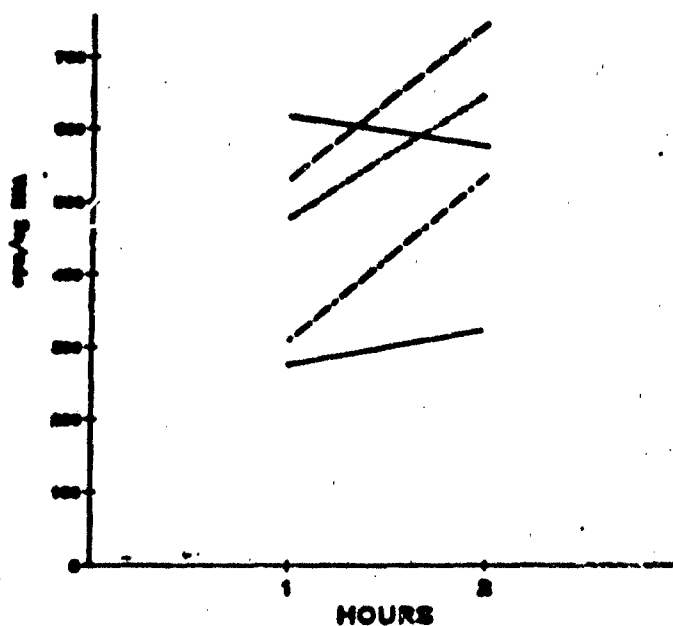


Figure 15. Incorporation of [^3H]thymidine after BCES treatment. Control (—); solvent control (---); exposure to BCES at 50 nmole/cm² (-.-.-), 100 nmole/cm² (- - - -), and 200 nmole/cm² (.....). Lines connect data points obtained at 1 and 2 hr, respectively.

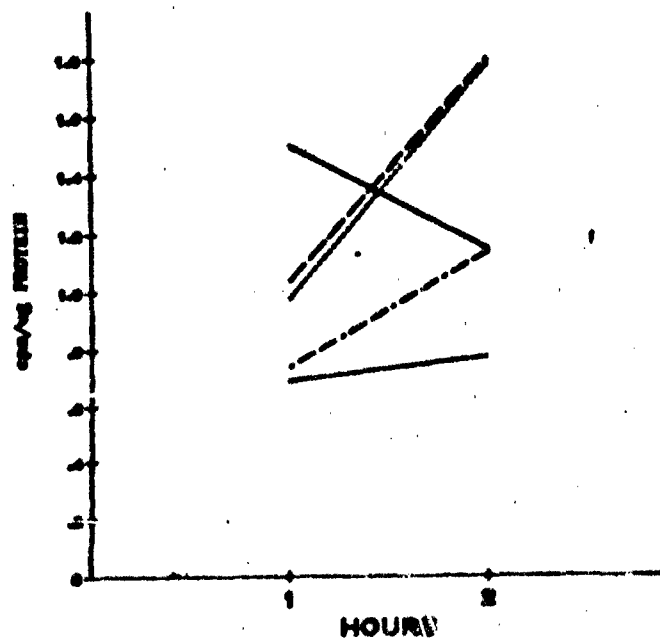


Figure 16. Incorporation of [^{14}C]leucine after BCES treatment. Control (—); solvent control (---); exposure to BCES at 50 nmole/cm² (.....), 100 nmole/cm² (-.-.-), and 200 nmole/cm² (- - - -). Lines connect data points obtained at 1 and 2 hr, respectively.

are not definitive. Replication of this work is in process.

b. Alkylation of DNA by BCES

As noted in section B, alkylation of DNA is likely to be the most important molecular lesion with which the exposed cell must deal if it is to survive. Studies on this phase of the work have therefore attempted to determine the degree of alkylation and cross-linking which occurs in cultures of keratinocytes exposed to BCES. Since one of the objectives of this project is to evaluate the role of this lesion in the necrosis of the exposed cell, low levels of BCES have been used in this work.

Submerged cultures grown in medium containing 0.08 mM Ca^{2+} , have been exposed to 16-64 μM BCES for 1 hr after which the cells were lysed by exposure to sodium dodecyl sulfate (SDS) for 1 hr at 37°C . After cooling to room temperature, the solution was made 0.25 M with respect to NaOH and incubated at room temperature for 20 min, which was sufficient to produce single strand breaks. Subsequent centrifugation in a gradient of alkaline sucrose produced a dose-related profile of single strand breakage in the DNA (Fig. 17). The DNA from cultures exposed to 64 μM BCES appeared at the top of the gradient. Decreasing the exposure from 64 through 16 to 0 μM resulted in banding of the DNA progressively toward the bottom of the gradient. Although it is not possible to calculate the number of alkylations per unit of DNA, these data clearly show that exposure to 64 μM BCES results in too much damage to make that level of exposure useful. In fact, future studies need not use concentrations greater than 16 μM and may require even lower levels of the mustard.

c. Development of an assay to measure fidelity in DNA repair

The use of viruses to investigate DNA repair has become widespread (35). In most such investigations, some modification of the technique of "host cell reactivation" has been applied. In principle, this experimental technique involves infection of a permissive host with a virus which is nonvirulent because of damaged DNA. The method depends on cellular repair processes to reverse the damage and reactivate the virus to virulence. This technique is to be used to determine the efficacy of the host cell's repair system and to evaluate the possible effects of a toxic chemical on the repair process. By inserting a specific lesion into the viral DNA, it should be possible to evaluate the repair system for that particular type of lesion. This technique is being developed for use in evaluating the effect of exposure to BCES on the "error free" repair capability of exposed keratinocytes.

Specifically, the probe will be a heteroduplex of simian virus 40 (SV 40) DNA containing two mismatched base pairs which confer temperature sensitivity on the large T antigen genome. This DNA will be used to transfect a culture of human keratinocytes which is semipermissive for SV 40. Repair will then be allowed to occur at a nonpermissive temperature so that only the repaired DNA, i.e., the non-temperature-sensitive genome, will be transcribed and translated to form the large T antigen. T antigen must be made in order for the viral DNA to be

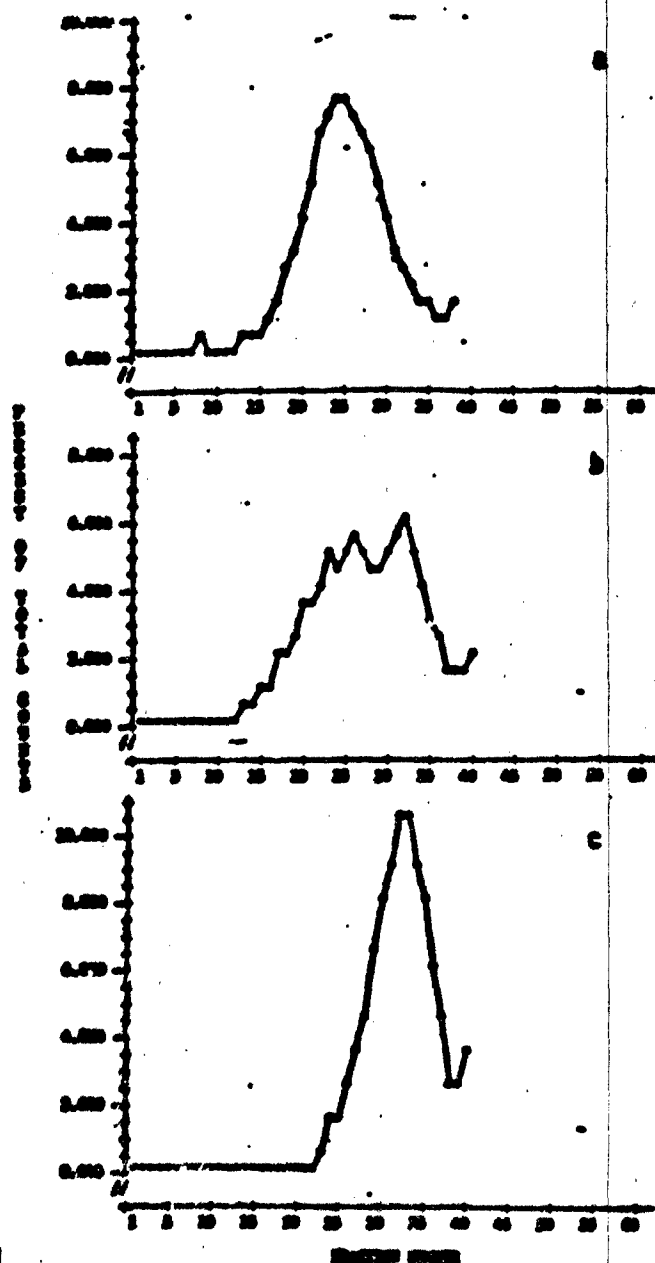


Figure 17. Alkaline sedimentation profiles of DNA in sucrose gradients, showing the appearance of single strand breaks after exposure to BCES. Three-day-old keratinocytes, grown in low calcium medium and prelabeled with [3 H]thymidine, were exposed to BCES at concentrations of 0 (a), 16 (b), and 64 μ M (c) for 1 hr. Cells were lysed with SDS, incubated for 1 hr at 37 $^{\circ}$ C and were then subjected to centrifugation in a gradient of alkaline sucrose. Procedural details are given in the text.

replicated. When T antigen is made from temperature-sensitive genome, the protein is not stable and viral DNA is not replicated. Repair of this damaged DNA will be observed by noting the time it takes for the host cells to repair the DNA and the percentage of transfected cells which carry out the repair. The end point assay will be the appearance of new viral DNA (determined by gel electrophoresis) or the appearance of viral plaques.

To date, stocks of wild type SV 40 virus have been prepared and titered by the plaque formation technique and viral DNA has been isolated and purified according to the Mirt procedure (36). Transfection of BSC-1 monkey kidney cells, the permissive host, has been achieved using indirect fluorescent antibody detection of T antigen as end point (37). Human cutaneous fibroblasts in culture have also been successfully transfected by the calcium co-precipitation technique (38). The percentage of transfected cells in both cases are much less than was the case with infection of the monkey kidney cells. Human keratinocytes have not yet been transfected in this laboratory although it has already been done in another laboratory (39).

6. Effect of BCES on mitochondrial integrity

BCES could interfere with mitochondrial function (cf. section B above). Inhibition of oxidative phosphorylation would be expected to initiate a sequence of reactions detrimental to the function and survival of the keratinocyte.

The rate of lactate accumulation by cultured cells may increase when oxidative metabolism is inhibited (40-42), because the normal route of lactate oxidation via oxidation of pyruvate and the tricarboxylic acid cycle has been blocked. The rate of lactate accumulation may also be increased as the rate of glycolysis is increased to supplement the production of ATP by extramitochondrial routes. Therefore, production of lactate by cultured keratinocytes could be a method of indirectly determining whether exposure to BCES results in an inhibition of mitochondrial function in these cells.

Confluent 9-day old cultures produced lactate in a linear manner for at least 10 hr (Fig. 18). Addition of dinitrophenol (50 μ M) in a 4:1 mixture of ethanol and methylene chloride increased the rate of lactate accumulation as compared with the control. The ethanol/methylene chloride mixture itself appeared to account for a small amount of the increase. During the first four hours of incubation after exposure to between 10 and 300 μ M BCES for 15 min, there did seem to be a decrease in the rate of lactate accumulation by submerged cultures as the level of mustard was increased (Fig. 19). After that time, the rate of accumulation of lactate showed an obvious decrease at all levels of exposure. Neither BCES at concentrations below 100 μ M nor methylene chloride at any concentration used appeared to affect the accumulation of lactate production. The data from this experimental approach suggest either that the rationale for these experiments was incorrect or that mitochondrial function is not strongly affected by exposure of keratinocytes to the concentrations of BCES which were used.

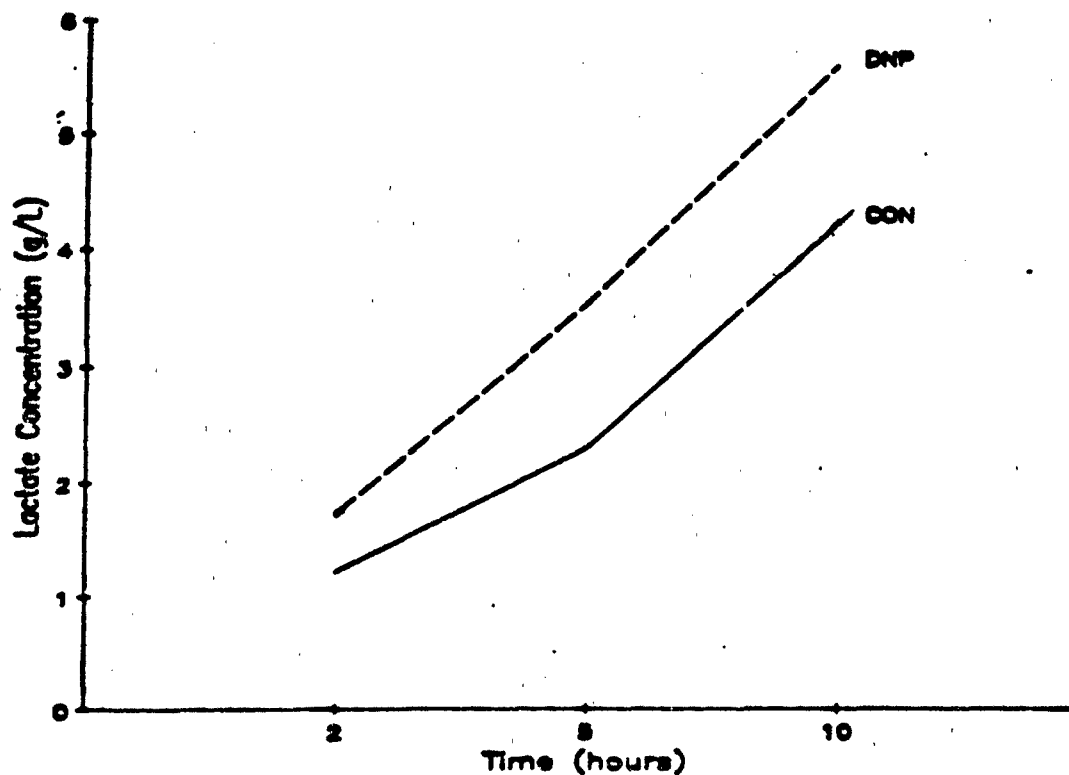


Figure 18. Lactate production by 9-day-old cultures of rat keratinocytes was stimulated by 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, in a 4:1 ethanol/methylene chloride mixture as compared with aqueous controls (CON). Basal cells were plated in 35-mm dishes, grown submerged in medium containing 1.8 mM Ca^{2+} , and exposed to 50 μM DNP for 10 hr. Each time point represents an average of a duplicate determination on each of three samples. Addition of the ethanol/methylene chloride mixture to the control stimulated lactate production slightly.

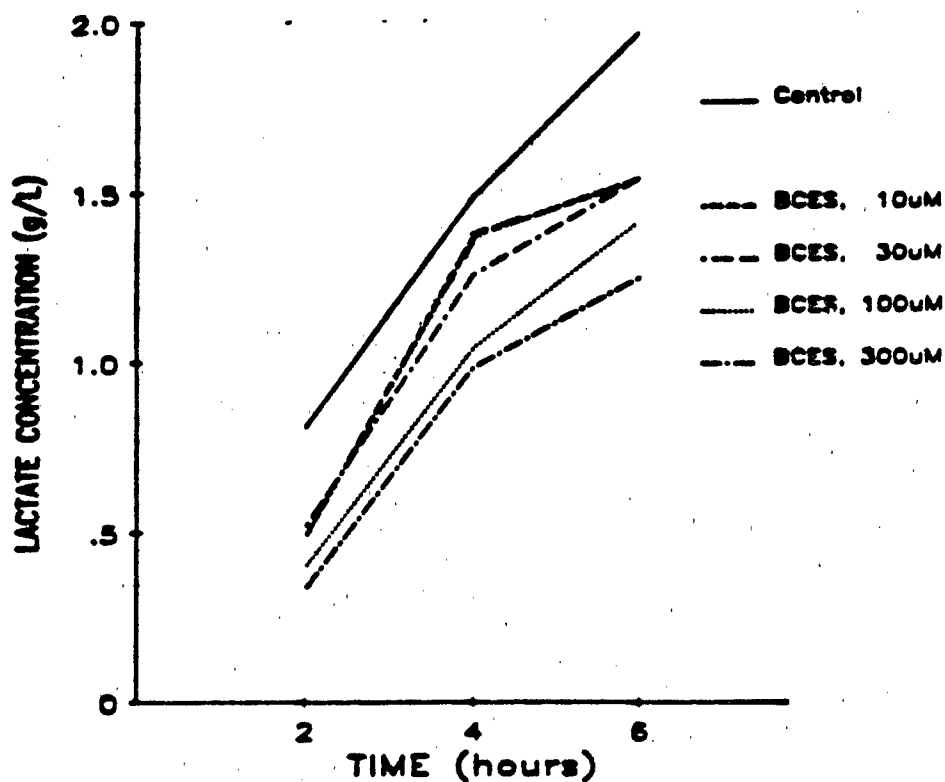


Figure 19. Effect of BCES on lactate production by 9-day-old rat keratinocyte cultures. Cultures were exposed to BCES in methylene chloride for 10 min. In this typical experiment, three dishes of cells were used for each exposure and duplicate samples were withdrawn for determination of lactate. For growth conditions of cultures, see legend for Figure 18. Control contained methylene chloride at 0.5% as did all reaction mixtures. This amount of methylene chloride had no effect on lactic production of control cultures.

In a further effort to determine a metabolic effect of exposure to a low level of BCES on keratinocytes in culture, protein synthesis was studied. The technique involved the incubation of proliferating cultures with [³H]leucine (10 uCi/plate; 180 Ci/mole) for 30 min followed by the addition of 10% trichloroacetic acid to stop the reaction and precipitate the protein which was collected on filters and processed for determination of radioactivity (43). BCES at concentrations up to 100 uM had no inhibitory effect on the incorporation of radioactive leucine in the cultures although the data showed considerable variability (Table 4).

The utilization of glucose was also studied. BCES also appeared to increase glucose consumption (Table 5). Concentrations of BCES between 10 and 100 uM appeared to increase the utilization of the sugar but 300 uM BCES had a slightly lower effect than did 100 uM BCES.

TABLE 4

Effect of Low Concentrations of BCES on Protein Synthesis

Treatment (uM BCE)	cpm/Plate	% Control
None	1,323 ± 388	100
10	800 ± 377	64
30	1,510 ± 535	114
100	1,001 ± 121	76
300	254, 260	20

Nine-day-old cultures of basal cells were exposed to BCES dissolved in EBSS for 15 min. The medium was withdrawn and replaced with fresh medium. After 6 hr of further incubation, the cultures were rinsed and the medium replaced with EBSS containing 4.0 uCi of [³H]leucine/ml. After 30 min, 10% trichloroacetic acid was added and the acid-precipitable radioactivity was determined. Values above are averages of 3 experiments, 3 samples, per experiment except for the last line of the table in which n=2. For growth conditions of cultures, see legend for Figure 12.

TABLE 5

Effect of Low Concentrations of BCES on the Rate of Glucose Consumption

BCES Concentration (μ M)	Glucose Consumption (μ mole/L/hr)
Control	0.047
Control + methylene chloride/ethanol solvent for BCES	0.066
20 (in methylene chloride/ ethanol as solvent)	0.127
50	0.169
100	0.255
300	0.231

The concentration of glucose was measured colorimetrically in 9-day-old cultures treated with BCES as was described for Table 4. For growth conditions, see legend for Figure 19.

It is, of course, possible that a metabolic abnormality may be a secondary contributor to the cellular necrosis associated with exposure to BCES *in vivo*. The reality of this possibility may well be defined in later experiments of this project when experiments will be done to determine whether the lesion in DNA caused by exposure to a low concentration of BCES is sufficient to cause cellular necrosis in the culture.

Because mitochondrial function may be inhibited as a secondary effect of BCES exposure, it was necessary to determine whether mitochondrial function could be directly inhibited. Therefore, the effect of BCES on the respiration of mitochondria from rat liver was determined. Rat liver was used rather than epidermis because of the availability of larger amounts of material from the liver. As far as is known, oxidative phosphorylation is comparable in the two tissues.

Since concentrations of BCES less than 250 μ M did not inhibit the rate of respiration of mitochondria from rat liver with succinate and glutamate as substrates, it seems unlikely that mitochondrial dysfunction is a major concomitant of early toxicity from this chemical.

The action of BCES on the integrity of mitochondrial membranes *in situ* was examined using the localization of Rhodamine 123 as the assay. This substance binds to energized mitochondrial membranes (44) and the mitochondrial profiles can be visualized by epifluorescence at appropriate wavelengths and high magnification in the light microscope. The dye diffuses from the mitochondria when the membrane is de-energized, e.g., when the mitochondria are uncoupled (45).

Preliminary results have been obtained with Rhodamine 123 staining of basal cell cultures. Cultures have not been exposed to BCES at this time. The Rhodamine-stained basal cells display a bright area of staining surrounding the nucleus that at high magnification appears as a fine, dense filigree of fibers that becomes more diffuse toward the margin of the cell. All cells do not appear to be uniformly brightly stained, although all cells do stain. The degree of staining may be a result of differentiation which is presumed to be different for each cell in the 9-day-old basal submerged culture maintained in the low calcium medium. Application of an uncoupler (e.g., 0.5 μ M p-trifluoromethoxy phenylhydrazine) to the culture causes the fluorescence to lose its localized pattern and to become more diffuse throughout the cytoplasm. A diminution of total fluorescence may also be present, although we cannot calibrate the quantum yield of the dye with the present instrumentation and software.

e. Development of techniques to study metabolism of BCES

During the term of the previous support, the activities of two enzymes which could play a role in the metabolism of BCES were examined in subcellular fractions prepared from whole skin, dermis, and epidermis of 4-day-old rats. The strain of rat was the same as that used for the cultivation of keratinocytes. The results of a study *in vivo* by Davison et al. (12) suggested that the initial glutathione-BCES adduct undergoes further degradation to generate different metabolites. Using optimal assay conditions, the presence of several enzymes was established (Table 6).

Glutathione S-transferase (GST) was assayed using 1-chloro-2,4-dinitrobenzene as a model substrate (46). Separation of dermis from epidermis was achieved by a modification of the method of Epstein et al. (47). Measurable GST activity was found in both the dermis and the epidermis (Table 7), with the largest percentage of the enzyme in the dermis. The sum of the activities in the dermis and the epidermis were consistently found to be greater than that found in the whole skin. The explanation for this effect was not investigated. Using a new rapid HPLC method in combination with affinity chromatography, substantial purification of this enzyme was achieved from the whole skin (Table 8).

Peroxidase was assayed using guaiacol (O-methoxyphenol) as a model substrate (48). This enzyme was predominantly associated with the nuclear and mitochondrial fractions derived from whole skin (Table 9). An approximately 80-fold purification of this enzyme was achieved starting with a whole skin homogenate (Table 10).

TABLE 6

Activities of Some Marker and Xenobiotic Metabolizing Enzymes in Subcellular Fractions of Cutaneous Cells

Enzyme/fraction	Enzyme activity (nmol/min/mg protein)
Lactic dehydrogenase (cytosol)	1390
Alcohol dehydrogenase (cytosol) with NAD ⁺	3.3
Acetaldehyde dehydrogenase (mitochondrial)	
with NADP ⁺	3.6
with NAD ⁺	7.2
Acetaldehyde dehydrogenase (microsomal)	
with NADP ⁺	23.0
with NAD ⁺	12.0

Tissue was homogenized in 0.05 M Tris buffer, pH 7.2 containing 0.25 M sucrose and submitted to successive centrifugation at 39,000 x g for 30 min at 4°C to sediment the mitochondrial fraction and 100,000 x g to sediment the microsomes. The supernatant solution from the last centrifugation was used to represent the cytosolic fraction. An increase in absorbance at 340 nm resulting from the production of NADH or NADPH was monitored. Lactate, ethanol, and acetaldehyde were used as the substrates. A millimolar extinction of 6.22 for the reduced form of the pyridine nucleotide was used to calculate enzyme activity.

TABLE 7

Distribution of Cytosolic GSHT_r Activity in Neonatal Rat Skin

Fraction	Volume (ml)	Protein (mg/ml)	Total (mg)	GSHT _r Activity		
				Specific Activity (nmol/min/ mg)	Total Activity (nmol/ fraction)	% Distribution
Whole skin	32	0.098	35.14	48	1690	100
Dermis	48	1.028	49.34	47	2320	88
Epidermis	18	0.576	10.37	31	310	12

Three-day-old rats (n=10-20 each) were used. The epidermis was separated from the dermis according to Epstein et al. (47). The epidermis was homogenized in 0.50 M Tris buffer, pH 7.2 and centrifuged at 100,000 x g for 30 min at 4° to obtain the cytosolic fraction which was used for the assays. GSHT_r activity was assayed according to Habig et al. (46), using CDNB as a substrate. The data represent the results of a typical experiment. Similar results were obtained in two additional experiments.

TABLE 8

Purification of GSHT_r from Whole Skin of 3-day-old Rats

Fraction	Specific Activity (mol/min/mg)	Fold Purification	% Recovery
Crude cytosol	0.034 ± 0.007	1	100
Affinity chromatography	29.88 ± 7	1021 ± 367	106 ± 20
HPLC-Applied	12.5	1	100
-Recovered	35.0	2.8	122

GSHT_r activity was assayed according to Habig et al. (46), using CDNB as a substrate. The data represent mean ± S.E. (n=3-7) or the results of a typical experiment. The cytosolic fraction (100,000 x g supernatant solution) was obtained by separating the dermis and epidermis (47), homogenizing the epidermis in 0.05 M Tris buffer, Ph 7.2 and centrifuging the homogenate at 100,000 x g for 30 min at 4° C. The active fraction was eluted with 10 mM GSH, pH 9.4, concentrated by ultrafiltration and further purified by HPLC using an anion exchange column.

TABLE 9

Subcellular Distribution of the Cutaneous Peroxidase Activity in 3-day-old Rats.

Subcellular Fraction	Peroxidase Activity (units/mg)	% Distribution
Cytosol	0.000	0.00
Nuclear	0.171	36.90
Mitochondria	0.263	57.18
Microsomes	0.025	5.92

Peroxidase activity was measured according to Himmelhoch et al. (48) using guaiacol as a substrate. One enzyme unit is defined as the amount of enzyme that causes a change of 1.0 absorbance unit per min at 470 nm. The results are mean peroxidase activity observed in two separate experiments. Each enzyme preparation represents pooled material from 20-30 rats. The cellular fractions were obtained by centrifugation of the tissue homogenate (in 0.05 M Tris buffer, pH 7.2 containing 0.25 M sucrose) at 10,000 x g for 30 min at 4° C to sediment the nuclear fraction, at 39,000 x g for 30 min to sediment the mitochondrial fraction, at 100,000 x g for 60 min to sediment the microsomal fraction leaving the final supernatant solution as the cytosolic fraction.

TABLE 10

Purification of Neonatal Rat Skin Peroxidase.

Fraction	Specific Activity (units/mg protein)	Fold Purification	% Recovery
Crude extract	0.29	1	100
Concanavalin A	7.17	24.7	70
Bio-Gel P-150	24.00	82.0	15

The data represent the results of a typical experiment. A homogenate of epidermis from 4-day-old rats, made in 0.50 M Tris buffer, pH 7.2, containing 0.25 M sucrose, was sedimented at 39,000 x g for 30 min at 4° C. The particulate fraction was suspended in 50 mM Tris, Ph 7.2 containing 0.25 M sucrose and 0.5 M CaCl₂ to solubilize the peroxidase which was recovered by centrifugation. The supernatant solution was subjected to affinity chromatography on a column of Concanavalin A-Sepharose. The adsorbed enzyme was eluted in a gradient of manitol. The eluted peroxidase was further purified on a column of Bio-Gel P-150. Analysis of the enzyme was carried out by the method of Himmelhoch et al. (48).

f. Protection against BCES-mediated damage of DNA through repair

BCES-mediated damage to DNA and repair thereof were measured by the alkaline unwinding/hydroxylapatite chromatographic assay (49).

Figure 20 shows that challenge of keratinocytes with 5 or 10 μM BCES for 30 min causes an initial increase in the percentage of the DNA which is double-stranded. This is taken to represent cross-linking. This figure shows that the fate of crosslinking over time differs dramatically between the 5 and 10 μM exposure concentrations. With a 5 μM concentration, cross-linking at time zero, i.e., immediately after BCES challenge, is repaired by 12 hr after exposure. On the other hand, with the 10 μM exposure, the cross-linking apparent at time zero changes into single strand breaks/alkali lesions at later time points. Moreover, this decline in percent double strand DNA continues throughout the course of the experiment, suggesting that the 10 μM concentration is causing cell death. These data indicate that keratinocytes have some capacity to survive BCES-induced DNA cross-linking but that this capacity is limited and can be relatively rapidly overwhelmed.

Table 11 shows that pretreatment of the keratinocyte culture with a non-DNA-damaging concentration of MNNG (0.05 μM) alters the pattern of DNA repair after challenge with 20 μM BCES. In control cells, cross-linking apparent at time zero after challenge changes into single strand breaks/alkali-labile lesions at later times. In MNNG-pretreated cells, on the other hand, the cross-linking is much more persistent and only resolves into single strand breaks/labile lesions somewhere between 6 and 24 hr after challenge. We have not yet determined the significance of this effect for BCES toxicity, nor its mechanism.

When submerged cultures, grown in medium containing 0.08 mM Ca^{2+} (consisting of 60% basal and 40% early differentiated cells) and cultures grown in the presence of 1.8 mM Ca^{2+} (consisting of a large fraction of late differentiated cells as well as basal cells), were exposed to 50 μM BCES for 30 min, washed, and assayed for the percentage of double-stranded DNA at zero time and after 4 hr of subsequent incubation, no differences were noted between the two culture types. Similarly, the effects of MNNG on the DNA were not different whether the cultures consisted of mostly germinative or differentiated cells.

Polyamines supplementation of the keratinocyte culture appears to prevent formation of strand cross-links (Fig. 21). (This assay does not distinguish between DNA-DNA and DNA-protein cross-links.) Ten micromolar BCES caused significant cross-linking of DNA, which is evident by a 12-16% increase in double strandedness. Pretreatment with three different polyamines appears to have eliminated this effect. Instead, single strand breaks/alkali-labile lesions are evident immediately after BCES challenge.

8 DS-DNA

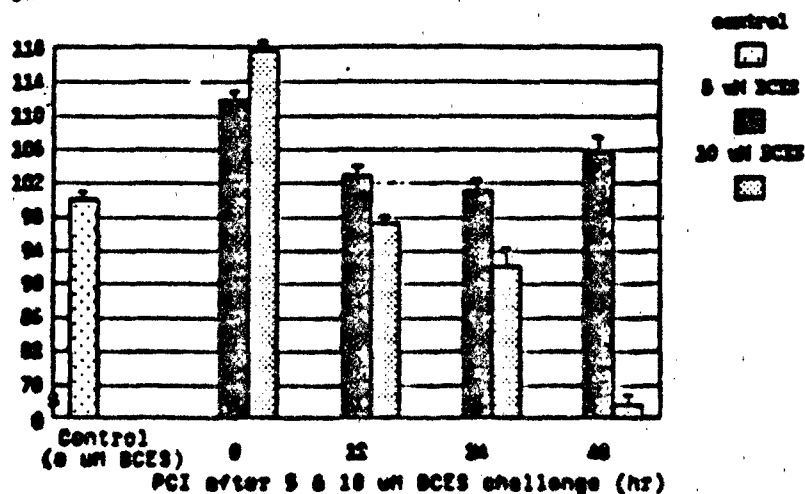


Figure 20. DNA damage induced by 5 or 10 uM BCES: studies of changes in percent DS-DNA by post-challenge incubation. Nine-day-old cultures grown submerged in medium containing 1.8 mM Ca^{2+} were used. Other growth techniques were as described by Brown et al (33). Exposure time was 30 min. PCI, post-challenge incubation. DS-DNA, double stranded DNA. Quantification of damage to DNA was carried out using the alkaline unwinding assay (49) for 1 hr at room temperature in the dark at pH 12.6, followed by chromatography on hydroxylapatite and fluorometric analysis of the eluted double stranded DNA. For purposes of graphing the data, the DS-DNA from unexposed cells was set at 100%. Error bars indicate the standard error (S.E.). $n=4$ for the control group and $n=6$ for BCES-exposed groups in two separate experiments. S.E. was $<3\%$. The 48-hr-value for 10 uM BCES is <4 .

TABLE 11

Protection Against BCES-mediated Damage by Prior Exposure to Nontoxic Level of MNNG^a

BCES challenge (20 μ M)	No.	Duration of postchallenge incubation (hr)	Pretreatment control (vehicle only) % control double-stranded DNA	MNNG Pretreatment % control double-stranded DNA
No	3		100 ^b	100 ^c
Yes	3	0	118 \pm 4	113 \pm 5
Yes	3	2	76 \pm 2	106 \pm 5
Yes	2	6	96	109
Yes	3	24	78 \pm 5	63 \pm 2

^a For growth conditions see legend for Figure 20. Assay for double stranded DNA was carried out as described by Kanter and Schwartz (49). Percentages are given \pm standard deviations.

^b 58 \pm 6 of DNA double-stranded.

^c 49 \pm 3 of DNA double-stranded.

8 DS-DNA

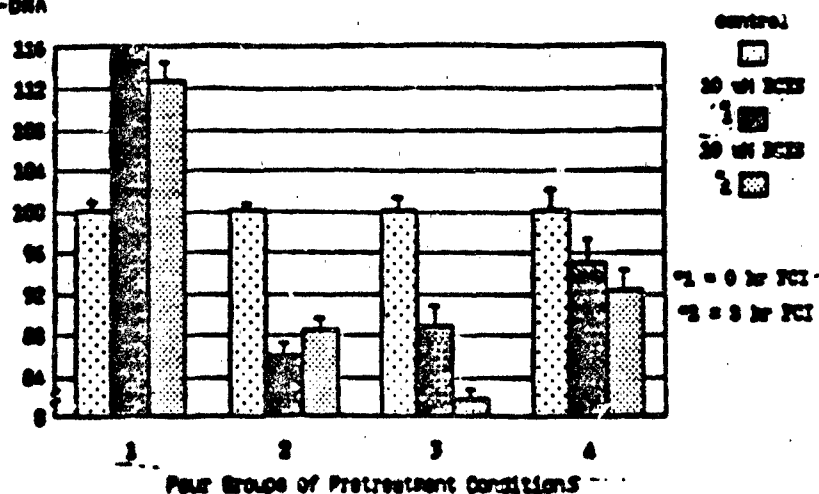


Figure 21. DNA damage and post-challenge incubation induced by 10 uM BCES: studies of polyamine treatment. Nine-day-old kkeratinocyte cultures, grown as described in the legend for Figure 20, were divided into the following groups: 1, control (no pretreatment); 2, pretreated with 20 uM putrescine from Day 4-8 of culture; 3, pretreated with 10 uM spermidine from Day 4-8 of culture; 4, pretreated with 10 uM spermine from Day 4-8 of culture. Cultures were treated with 10 uM BCES for 30 min, washed one time with phosphate-buffered saline, pH 7.4, containing dextrose and further incubated for 3 hr in complete growth medium. Analyses for DS-DNA was carried out by the alkaline unwinding assay (48) followed by chromatography on hydroxylapatite and fluorometric analysis of DNA. Error bars indicate S.E. n=4 for control and n=6 for BCES-exposed cells from two separate experiments. S.E. <5%.

E. Summary of Findings

1. Obtaining stratified terminally differentiating cultures

Using the protocols developed in this investigation, it is possible to consistently produce stratified terminally differentiated primary cultures of cutaneous keratinocytes from the epidermis of the newborn rat. The procedure is also applicable to human keratinocytes derived from newborn foreskin although the rate of growth and differentiation is considerably slower than is the case with murine cells. All biochemical and morphological parameters investigated to date are similar in the cultures as they are *in situ*. Physically, the cultures have a surface topography and a consistency which allows them to be exposed to BCES topically and to be manipulated for subsequent analysis.

The stratified cultures can be exposed to BCES dissolved in 70% DMSO. It appears that the BCES attacks the lower cells by passing through the tissue rather than around it into the medium but this has not been proven as yet.

2. Proliferating monolayers of keratinocytes

Growing keratinocytes in low calcium medium produces cultures which remain as monolayers although cells in the monolayer do exhibit early steps in differentiation. This culture is particularly useful for studying the direct interaction between BCES and the cell since the toxicant is added to the medium in which the cells are immersed.

3. Purified populations of basal and differentiated cells

Purified populations of basal cells can be obtained by sedimentation in a density gradient of Percoll. By morphology, such preparations are 98% pure but lectin binding techniques indicate that biochemically, this population consists of proliferating as well as early differentiated cells.

4. Dose response curves indicate that the integrity of DNA is compromised immediately when cells grown submerged in 0.08 mM Ca^{2+} , are exposed to 16 μM BCES. It seems likely that an even lower exposure would affect the integrity of DNA. This level of exposure to BCES does not appear to produce metabolic effects until several hr after exposure. Higher exposures (e.g., greater than 100 μM BCES) are required to dramatically affect glycolysis, glucose utilization, protein synthesis, RNA synthesis, mitochondrial respiration or ultrastructural morphology.

5. Glutathione S-transferase and peroxidase, two enzymes which might influence the toxicity of BCES by inactivating the toxicant, were identified as present in the epidermis and were purified from whole skin.



F. Recommendations

The biological systems developed in this project are appropriate for use in determining the molecular and cellular mechanisms responsible for the toxic manifestations associated with vesiculation. Attention should now be focused on determining the most sensitive indicator of toxicity in order to define the nature of the molecular interactions which lead to toxicity from BCES. In this context, the keratinocyte's ability to repair molecular and cellular damage done by BCES should be evaluated. The objective of further study should be to develop a profile of molecular and cellular alterations in relation to increasing levels of exposure to BCES. This kind of information should provide an understanding of the kinds of BCES-mediated effects which the cell can accommodate and identify those which exceed its ability to tolerate leading to overt toxicity.

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